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(54) Title: EXPRESSION OF FUNCTIONAL VERTEBRATE PHOSPHOLIPASES IN YEAST

(57) Abstract

Yeast cells comprising a heterologous phospholipase C protein and methods for their use in drug screening assays to identify a modulator (i.e. agonist or antagonist) of a phospholipase C are described.

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Expression of Functional Vertebrate Phospholipases in Yeast

Cross Reference to Related Applications

This invention is a Continuation-in Part of U.S.S.N 08/431,632, filed on June 7, 1995.

Field of the Invention

This invention relates to functional expression of heterologous phospholipases in yeast, the recombinant yeast cells, and methods for using the engineered cells, including, inter alia, the identification of agonists and antagonists of signal transduction pathways which involve phospholipases.

Background of the Invention

Eukaryotic phospholipases are known to be involved in cellular signal transduction processes important in regulation of cellular growth, differentiation and secretory events in numerous cell types. The binding of agonists to certain cell surface receptors leads to the generation of a variety of signaling molecules which include phospholipid derived second messengers. The phospholipid-derived second messengers mediate both transient and sustained responses and are implicated in a wide variety of physiological responses including growth regulation, immune modulation and neurotransmission. Studies over the last two decades indicate that membrane lipids serve as substrates for the variety of different phospholipases that are responsible for the generation of phospholipid derived second messengers. These include phosphatidylinositols (the substrate for phospholipase C enzymes, see below), phosphatidylcholine, phosphatidylethanolamine, sphingolipids (e.g. sphingomyelin, sphingosine, sphingosine phosphate and sphingosine phosphocholine). See Dicheva and Irvine (1995) Cell 80:269-278 for a recent review.

There are at least five distinct types of phospholipase activities known to be responsible for the generation of phospholipid second messengers, namely phospholipase A (including phospholipases A_1 and A_2), phospholipase B, phospholipase C, phospholipase D and sphingomyelinase. The distinguishing feature of each of these phospholipases is the substrate bond cleaved, and hence the second messengers derived from a given phospholipid substrate.

PLA_1 and PLA_2 (E.C.3.1.1.4) catalyze the release of fatty acids, in particular arachidonic acid (AA), from the *sn*1 and *sn*2 positions of 1,2-diacylglycerol, 3-phosphocholines, generating AA and lysophosphorylcholine (lyso-PC) respectively. AA, in

turn, has at least four known metabolic fates, namely, generation of proinflammatory eicosanoids (leukotrienes and prostaglandins), reincorporation into existing membrane phospholipids, reincorporation into soluble phospholipid metabolic pathways, and direct secretion from cells. In addition, lyso-PC serves as the precursor for the production of lipid second messengers like platelet activating factor (PAF). cDNA and genomic clones encoding the pancreatic form of PLA_2 have been isolated from a variety of eukaryotic sources including bovine (Tanaka et al. 1987), rat (Ohara, O. et al. 1986 and 1990; and Kusunoki, et al. 1990), dog (Kerfelec et al. 1986),and pig (Seilhamer et al. 1986). In addition to the pancreatic PLA_2 isoform, a variety of cellular PLA_2 ($cPLA_2$) isoforms have been isolated from a wide variety of mammalian tissues and cell types including brain (Gray and Strickland, 1982), liver (DeWinter et al. 1982), spleen (Teramoto et al. 1983), macrophages (Trotter and Smith (1986) Neurochem. Res. 11:349-361), leukocytes (Traynor and Authi 1981), and others (for a review see Ban Den Bosch, 1980). Clones for human (Clark et al. 1991; Sharp et al 1991) and murine (Clark et al. 1991) $cPLA_2$ gene products have been isolated and characterized. The 85kDa gene product encoded by these cDNAs is expressed in cells types involved in the inflammatory response (neutrophils, platelets, monocytes and macrophages) and its expression is regulated by, for example, tumor necrosis factor, IL-1, TGF-β, MCSF and glucocorticoids.

Phospholipase B (PLB) has been described in a variety of eukaryotic species from fungi to mammals. PLB isoforms have several catalytic properties including the release of fatty acids from the *sn*1 and *sn*2 positions of phospholipids (as with PLA_1 and PLA_2), the release of fatty acids from lysopholipids, and in the yeast *Saccharomyces cerevisiae*, acyltransferase activity that catalyzes the synthesis of phospholipids from lysophospholipids (Kuwabara et al. 1989; Gassama-Diagne 1989; and Lee et al. 1994). Genomic clones for PLB-like enzymes have been isolated from several sources including *S. cerevisiae* (Lee et al. 1994) and *Penicillium notatum* (Masuda et al. 1991).

Phospholipase C enzymes (PLC; E.C. 3.1.4.3), of which there are at least four subfamilies known as PLC-α, -β, -γ, and -δ (For reviews, see Rhee et al. 1989, and Cook and Wakelam 1992b) catalyze the hydrolysis of phosphatidylinositol (4,5) bisphosphate (PIP_2) to form two second messengers, inositol 1,4,5-trisphosphate (IP_3) which is involved in the mobilization of intracellular stores of calcium, and diacylglycerol (DAG), which binds to and activates selected isoforms of protein kinase C (PKC; E.C. 2.7.1.37). This class of enzymes, and second messengers generated by them have been demonstrated to be linked to receptor tyrosine kinases, G-protein coupled receptors and to MIRRs like the T-cell antigen receptor, the B-cell antigen receptor and immunoglobulin Fc receptors.

Phospholipase D (PLD; E.C. 3.1.4.4) catalyzes the hydrolysis of phosphatidylcholines to yield phosphatidic acid (PA) and free choline (see Bishop, Pachter and Pai 1992; and Cook and Wakelam, 1992a). While neither PA nor free choline has been shown to function as a second messenger directly, it has been reported recently that PA or its lyso derivative, lyso-PA, may function to stimulate hydrolysis of polyphosphoinositides, activation of PKC, inhibition of adenylyl cyclase and stimulation of DNA synthesis (Moolenaar et al, 1986; Murayama and Ui, 1987, Van Corven et al, 1989; and Plevin et al, 1991b. refs in Cook and Wakelam, 1992b), as well as activate kinases directly in a manner similar to DAG (Eppand and Stafford, 1990, and Bocckina and Exton, 1990, refs in Cook and Wakelam, 1992b). In addition, phosphatidic acid formed by the action of PLD can be converted to DAG by the action of phosphatidic acid phosphohydrolase (E.C. 3.1.3.4), and thus serve as a secondary reservoir for the production of DAG by PLC-independent mechanisms (Bonser et al, 1989 cited in Divechia and Irvine 1995, review; and Cook and Wakelam, 1992b). cDNA clones for phospholipase D have been isolated from a number of different species including *Arcanobacterium haemolyticum* (Cuevas, W.A., and J.G.L. Songer, 1993), *Corynebacterium pseudotuberculosis* (unpublished, GenBank Accession numbers L16586, L16587), *Vibrio damsela* (unpublished, GenBank Accession number L16584) and human (Tsang et al. 1993)

Phospholipase C enzymes

Phospholipase C enzymes (PLC; E.C. 3.1.4.3) are intracellular enzymatic mediators of a wide variety of cellular responses to extracellular stimuli. Binding of hormones, growth factors, neurotransmitters, and other agonists to specific cell surface receptors initiates the activation of PLC isozymes, which in turn result in the production of at least two well characterized active second messengers, inositol-1,4,5-trisphophate (1,4,5-IP_3) and *sn*-1,2-diacylglycerol (DAG). 1,4,5-IP_3 binds to a specific family of intracellular receptors localized on modified portions of the endoplasmic reticulum that bears homology to the ryanodine receptor family of intracellular calcium channels (the IP_3 receptor family, reviewed in Berridge, 1993). In addition to functioning as the IP_3 receptor, these receptors also function as IP_3-sensitive calcium channels which cause release of intracellular stores of calcium upon liganding with IP_3.

Increases in intracellular calcium, in turn, result in the transient or sustained activation of a variety of cellular signaling pathways which include but are not limited to activation of protein kinase C (PKC) isoforms, transcriptional activation through the Mitogen Activated Protein Kinase (MAPK) and Jun Kinase (JNK) pathways, and mitogenesis. DAG, on the other hand, forms a complex with phosphatidylserine and calcium and activates protein kinase C (PKC) isozymes, which in turn activate signaling pathways by direct

phosphorylation of a variety of substrates which include adenylyl cyclases and RAF-1 (P.J. Parker et al, 1986; Coussens et al. 1986a, and Kolch et al. 1993).

The division of PLCs into four classes, α, β, γ, δ, originally corresponded to immunologically distinct proteins of 56 kDa, 150 kDa, 145 kDa, and 85 kDa purified from bovine brain (PLC-β, -γ, and -δ) and other tissues including liver, seminal vesicles or uterus (PLC-α). (Described in Suh et al. 1986; and summarized in A.J. Morris et al 1990). All of these enzymes have similar catalytic properties in that they hydrolyze phosphatidyl inositol (PI), phosphatidyl inositol 4-monophosphate (PIP) and phosphatidyl inositol-4,5,bisphosphate (PIP_2) in a calcium dependent manner, but not phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine. However, these immunologically distinct isozymes are distinguishable in several regards, not the least of which is that they are all unique gene products encoded by different genes, as discussed in further detail below. At the enzymatic level, these enzymes differ with respect to both their catalytic properties and their modes of regulation. With respect to divalent cation requirements for activity, for example, polyphosphoinositides are the preferred substrates for the PLC-β and -δ classes at low or physiological intracellular calcium concentrations, while PLC-γ isozymes prefer PI and PIP_2 at physiological intracellular calcium concentrations. The modes of regulation of PLC isozymes also differ considerably with respect to one another. The PLC-β isozymes are activated in response to ligand binding to G-protein coupled receptors and are directly stimulated by Gα and Gβγ components of heterotrimeric G-proteins. PLC-γ isozymes, on the other hand, are activated directly by phosphorylation via receptor and non-receptor tyrosine kinases. While little is known about the regulation of PLC-α activity in vivo or in vitro, recent studies of PLC-δ regulation suggest that it may be regulated by a novel class of regulatory proteins (p122-RhoGAP) that show similarity to the GTPase activating protein homology region of bcr, display GAP activity towards RhoA, and bind to and directly stimulate PLC-δ1 (Homma, Y., and Y. Emori, 1995).

Cloning of PLC isozymes

Molecular cloning studies have resulted in the identification of at least ten genes encoding distinct PLC isozymes. Several PLC-α cDNA clones have been isolated from a variety of sources including rat basophilic leukemia cells (Bennett et al. 1988), mouse lymphocytes (Hempel and DeFranco, 1991), and bovine tissue (Hirano et al, GenBank Accession number D16235). Four PLC-β encoding cDNAs encoding subtypes -β1-4 (Suh et al 1988; Bahk et al. 1994; Park et al. 1992; Lee et al. 1993; and Kim et al. 1993) have been reported. Two PLC-γ encoding cDNAs have been reported, which correspond to PLC-γ1 and -γ2 subtypes (Emori et al. 1989; Suh, et al, 1988; and Burgess et al. 1990). Three PLC-δ

encoding cDNAs encoding subtypes PLC-δ1-3 have been published (reviewed in Cook and Wakelam, 1992b and Boyer et al, 1994).

Sequence Comparisons of PLCs

The overall primary structural identity between PLC-β isoforms is quite low (< 30% identity), in part explaining the differences in size among the different isozymes and the lack of immunological cross-reactivity observed with antisera and monoclonal antibodies developed against the various purified isozymes. PLC-β, -γ,and -δ isozymes share considerable sequence homology, however, in two regions, variously referred to as the X and Y regions (Rhee et al, 1989) or regions I and II (Meldrum, et al. 1991). The X region is approximately 150 amino acid residues in length, while the Y region is approximately 260 residues in length. Between classes, the X regions share approximately 50% identity, while the Y regions share approximately 40% identity in pairwise comparisons amongst PLC-β, -γ, and -δ isozymes. Based on the high degree of conservation of these two domains among the PLC-β, -γ,and -δ isozymes, it has been argued that these regions contribute to the catalytic activity of these enzymes. An additional feature of the spacer region between the X and Y regions is that in PLC-β, and -δ classes, these regions are separated by 50-100 residues rich in serine, threonine and acidic residues with no particular homology to known regulatory motifs, while in the PLC-γ class the X and Y regions are separated by more than 400 residues. This region of the PLC-γ isoforms encodes two src homology 2 (SH2) domains and one src homology 3 (SH3) domain, which are not present in either PLC-β or -δ enzymes. As discussed below, the SH2 domains are involved in regulation of PLC-γ activation by receptor and non-receptor tyrosine kinases.

Sequence analysis of the PLC-α cDNA clones from a rat basophilic leukemia cell line reveals considerable sequence divergence of this class of phospholipase C isozymes from the PLC-β, -γ,and -δ isozymes. PLC-α encodes neither the highly conserved X or Y regions found in all other PLC isoforms nor SH2 or SH3 domains found in PLC-γ isoforms.

Yeast PLC

In addition to the mammalian PLC-β isoforms discussed in the preceding sections, various PLCs have been isolated from several other organisms. Of considerable importance in this regard is the molecular cloning of the gene (PLC1) encoding a protein with homology to the PLC class of phospholipases from the yeast *Saccharomyces cerevisiae* (Yoko-O et al. 1993; Payne and Fitzgerald-Hayes, 1993; and Flick and Thorner, 1993). Sequence analysis of the independently identified clones reveals first that all three clones are from the same

genetic locus and that the protein coded for by this gene most resembles mammalian PLC-δ isozymes both with respect to size of the enzyme encoded by it and with respect to structural features such as the relative distance between the X and Y domains, the size of the carboxyl terminal extension beyond the Y domain, and the absence of SH2 and SH3 domains between the X and Y motifs (this last point is to be expected as yeast show no evidence of phosphotyrosine modification by biochemical analyses). Flick and Thorner (1993) overexpressed and purified this enzyme from yeast and demonstrated an associated phospholipase C activity with divalent cation requirement and substrate preferences that resemble those of mammalian PLC-δ isozymes. Finally, genetic analysis of *S. cerevisiae* strains with deletions at the PLC1 locus (*plc1*Δ) reveal a variety of conditionally lethal phenotypes associated with the *plc1*Δ genotype, such as, sensitivity to high osmolarity, poor growth on carbon sources other than glucose (i.e. galactose, raffinose, glycerol/ethanol), temperature sensitive growth at elevated temperatures (>35°C) and finally, chromosome missegregation (Flick and Thorner, 1993, and Payne and Fitzgerald-Hayes, 1993).

Expression of Foreign Proteins in Yeast Cells

A wide variety of foreign proteins have been produced in *S. cerevisiae*, that remain in the yeast cytoplasm or are directed through the yeast secretory pathway (Kingsman et al. TIBTECH 5, 53 (1987). These proteins include, as examples, insulin-like growth factor receptor (Steube et al. Eur. J. Biochem. 198, 651 (1991), influenza virus hemagglutinin (Jabbar et al. Proc. Natl. Acad. Sci. 82, 2019 (1985), rat liver cytochrome P-450 (Oeda et al. DNA 4, 203 (1985) and functional mammalian antibodies (Wood et al. Nature 314, 446 (1985). Use of the yeast secretory pathway is preferred since it increases the likelihood of achieving faithful folding, glycosylation and stability of the foreign protein. Thus, expression of heterologous proteins in yeast often involves fusion of the signal sequences encoded in the genes of yeast secretory proteins (e.g., α-factor pheromone or the SUC2 [invertase] gene) to the coding region of foreign protein genes.

A number of yeast expression vectors have been designed to permit the constitutive or regulated expression of foreign proteins. Constitutive promoters are derived from highly expressed genes such as those encoding glycolytic enzymes like phosphoglycerate kinase (PGK1) or alcohol dehydrogenase I (ADH1) and regulatable promoters have been derived from a number of genes including the galactokinase (GAL1) gene. In addition, supersecreting yeast mutants can be derived; these strains secrete mammalian proteins more efficiently and are used as "production" strains to generate large quantities of biologically active mammalian proteins in yeast (Moir and Davidow, Meth. in Enzymol. 194, 491 (1991). The following two reviews of the field are incorporated herein by reference in their entireties:

"The Molecular and Cellular Biology of the Yeast. *Saccharomyces*. Genome dynamics, Protein Synthesis, and Energetics." 1991. Edited by J.R.Broach, John Pringle and Elizabeth Jones. Cold Spring Harbor Laboratory Press; "Guide to Yeast Genetics and Molecular Biology" 1991. Methods in Enzymology vol. 194. Edited by Christine Guthrie and Gerald R. Fink, Academic Press.

Several groups have attempted to express phospholipases in yeasts. Yoko-o et al., ((1993) *Proc. Natl. Acad. Sci. USA* 90:1804) claim to achieve complementation of a yeast PLC defect with an uncoupled rat PLCδ1. Yoko-o et al. report the cloning of PLC from *S. cerevisiae* by PCR methodology using primers designed to incorporate sequences from two conserved amino acid sequences found in the X-region of mammalian PLC enzymes After cloning the gene, they proceeded to make gene disruptions at the PLC1 locus. Heterozygous diploid transformant (PLC1/plc1::HIS3) were sporulated and tetrad analyses were performed. In the TY1 background, all four spores were found to be viable at a range of temperatures including 18°C, 30°C, and 37°C. In the TY4 background, Yoko-o et al. observed lethality at 30°Cin tetrad analyses. These phenotypes are in contradiction to those described herein, and those reported by Flick and Thorner in the art, where conditional lethality at 37°C is observed. This is important because it is not possible to measure complementation of a growth defect under the nonselective conditions of the Yoko-o et al experiments. Thus, Yoko-o et al. do not observe functional integration of the rat PLCγ into the yeast cell.

Arkinstall et al ((1995) *Mol. Cell. Biol.* 15:1431) expressed a mammalian platelet-derived growth factorβ receptor and PLCγ2 in *Schizosaccharomyces pombe*. Arkinstall et al. found that in cells coexpressing the mouse PDGFβ receptor and PLCγ2, levels of [^{3}H]inositol phosphates in radioactively labeled yeast cells were increased. Coexpression of c-src was also found to result in phospholipid hydrolysis. Because these experiments only measure the immediate biochemical consequence of phospholipase Cγ stimulation, this work also fails to teach the functional integration of a mammalian PLCγ into a yeast cell.

Brief Description of the Figures

Figure 1 is a graphic representation showing human phospholipases and Gα16 complementation of the *plc1* growth defect on high salt medium: *S. cerevisiae* strain CY1630 (MATα plc1Δ1::His3 ade2-101 his3Δ200 leu2Δ1 lys2-801 trp1Δ1 ura3-52) transformed with plasmids carrying each of the following as described in the text was streaked out in sectors 1-6: (1) Gal1p-rat PLC-β1 and GPA1p-Gα16; (2) CUP1-human PLC-β2 and no G protein; (3) CUP1-human PLC-β2 and GPA1p-Gαi2-Q205L; (4) CUP1-human PLC-β2 and GPA1p-GαS-Q227L; (5) CUP1-human PLC-β2 and GPA1p-Gα16; (6) No PLC

and GPA1-Gα16. In the figure the symbol (+) represents growth and (-) represents no growth.

Summary of the Invention

The present invention relates to the functional expression of mammalian phospholipases in yeast cells. The use of the engineered yeast cells in identifying potential inhibitors or activators of the mammalian phospholipase, or of other proteins which belong to a phospholipase signal transduction pathway, i.e., proteins which are naturally or artificially "coupled" to the mammalian phospholipase in the engineered yeast cell is also within the scope of the invention. The term "coupled" here means that inhibition or inactivation of the coupled protein results in inhibition or activation (not necessarily respectively) of the phospholipase. The term coupled is meant to include both "natural" coupling of heterologous signaling molecules into a yeast signal transduction pathway and "artificial" coupling of heterologous signaling molecules into a yeast pathway in a manner not normally operative in yeast cells. Functional expression of human phospholipases is especially desirable.

In general, the yeast cells of the subject invention are characterized as including a heterologous gene encoding a heterologous phospholipase C (PLC), e.g., a polypeptide having a phospholipase C activity. In preferred embodiments, the PLC is a mammalian PLC such as a human PLC. For example, the PLC can be a phospholipase selected from the group consisting of PLCα's, PLCβ's, PLCδ's and PLCγ's. Preferred PLC's are of the β subtype, such as PLCβ1, PLCβ2, PLCβ3 and PLCβ4. In certain embodiments, the heterologous phospholipase C can be constutitively activated. This may occur by way of mutations to the PLC itself which result in a higher level of activity, relative to the wild-type protein, in the absence of signaling to the PLC. In other embodiments, the constitutive activation of the PLC can occur due to overexpression and/or consitutively activating mutations to upstream regulators of the PLC, such as receptors, G proteins and the like.

In certain embodiments, the recombinant cell is characterized by the ability of the heterologous PLC to hydrolyze phosphatidylinositol 4,5-bisphosphate. In other embodiments, the yeast cell has a loss-of-function mutation to an endogenous phospholipase gene, giving it a first detectable phenotype, and the heterologous PLC complements the loss-of-function mutation and confers a second detectable phenotype on the cell.

In preferred embodiments, the heterologous PLC is functionally integrated in a phospholipase-dependent signal pathway of the cell. For example, the heterlogous PLC

may complement a loss-of-function mutation of an endogenous phospholipase gene of the yeast cell, e.g., the heterologous PLC may complement the *plc1*Δ mutation. Expression of the heterologous PLC may confer a phenotype to the host cell which is detectably different than the phenotype of the host cell in the absence of the heterologous PLC protein. For instance, complementation of a loss-of-function mutation, such as the *plc1*Δ mutation, by the heterologous PLC can rescue/overcome such phenotypes as temperature-sensitivity and/or salt sensitivity. In certain embodiments, the heterolgous PLC, via a phospholipase-dependent signal pathway, can modulate such downstream effectors as calcium mobilization and/or PKC activity.

Moreover, in those embodiments wherein the phospholipase-dependent signal pathway modulates gene expression, the recombinant cell may also be engineered to include a reporter gene construct containing a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the phospholipase-dependent signal pathway, expression of the reporter gene providing the detectable signal. Exemplary reporter genes encode a gene product that gives rise to a detectable signal, such as color, fluorescence, luminescence, cell viability relief of a cell nutritional requirement, cell growth, or drug resistance. In preferred embodiments, the reporter gene encodes a gene product selected from the group consisting of chloramphenicol acetyl transferase, beta-galactosidase, luciferase, fluorescent protein, and alkaline phosphatase. In other embodiments, the reporter gene encodes a gene product which confers the ability to grow in the presence of a selective agent, e.g., canavanine.

In another embodiment the yeast cell further comprises a heterologous regulatory protein which is coupled to the phospholipase signaling pathway, e.g., the regulatory protein modulates the activity of the heterologous PLC or is modulated by the PLC activity. Exemplary regulatory proteins include, for example, G-protein heterotrimers; G α subunits; G$\beta\gamma$ subunits; proteins known to interact functionally with PLC substrates (e.g., profilin, gelsolin, cofilin, and alpha-actinin); and other effectors whose activity is dependent on PLC activity (i.e., IP_3 receptors/calcium channels, and IP_3 or calcium sensitive enzyme activities such as PKC or calcium/calmodulin kinase (CaM kinase)).

In another embodiment the yeast cell comprises a heterologous receptor or ion channel or other surface protein capable of transducing signals via the phospholipase-dependent signal pathway, e.g., which is known to couple to PLC activity either directly or via a regulatory protein. This aspect of the invention provides for more general readout systems to assay modulation of the activity of a variety of different cellular signaling components which effect phospholipase C activation. In a preferred embodiment, the host cell further comprises a heterologous gene encoding a heterologous G-protein coupled

receptor. Preferred G-protein coupled receptors include chemoattractant peptide receptors, neuropeptide receptords, cytokine, light receptors, neurotransmitter receptors, cyclic AMP receptors, and polypeptide hormone receptors. For example, the receptor may be any one of: an α1A-adrenergic receptor, an α1B-adrenergic receptor, an α1C-adrenergic receptor, an M_1 AChR receptor, an M_3 AChR receptor, an M_5 AChR receptor, a D_2 dopamine receptor, a D_3 dopamine receptor, an A1 adenosine receptor, a 5HT1-like receptor, a 5HT1d-like receptor, a 5HT1d beta receptor, a substance K (neurokinin A) receptor, a f-Met-Leu-Phe (FMLP) receptor, an angiotensin II type 1 receptor, a *mas* proto-oncogene receptor, an endothelin ETA receptor, an endothelin ETB receptor, a thrombin receptor, a growth hormone-releasing hormone (GHRH) receptor, a vasoactive intestinal peptide receptor, an oxytocin receptor, a SSTR3 receptor, an Luetinizing hormone/chorionic gonadotropin (LH/CG) receptor, a thromboxane A2 receptor, a platelet-activating factor (PAF) receptor, a C5a anaphylatoxin receptor, an Interleukin 8 (IL-8) IL-8RA receptor, an IL-8RB receptor, a mip-1/RANTES receptor, a metabotropic glutamate mGluR1-5 receptor, an ATP receptor, an amyloid protein precursor receptor, a bradykinin receptor, a gonadotropin-releasing hormone receptor, a cholecystokinin receptor, an antidiuretic hormone receptor, an adrenocorticotropic hormone II receptor, LTB4, LTD4 tachykinin receptor, thyrotropin releasing hormone receptor, and oxytocin receptor. In other embodiments, the the receptor protein is a receptor tyrosine kinase.

In yet another embodiment, the recombinant cells are further engineered to include an expressible recombinant gene encoding a heterologous test polypeptide. In preferred embodiments, such cells are provided as mixed culture collectively expressing a variegated population of test polypeptides.

The present invention also relates to a rapid, reliable and effective assay for screening and identifying pharmaceutically effective compounds that specifically interact with and modulate the activity of a phospholipase. The subject assay enables rapid screening of large numbers of test compounds to identifying those compounds which modulate, e.g., agonize or antagonize, phospholipase bioactivity. In general, the assay is characterized by the use of the recombinant cells described herein as engineered to express heterologous phospholipase activities which produce, in the engineered cell, a detection signal. The ability of particular test compounds contacted with the instant cells to modulate the phospholipase activity can be scored for by detecting up or down-regulation of the detection signal. For example, second messenger generation via the heterologous phospholipase activity can be measured directly. Alternatively, the use of a reporter gene can provide a convenient readout. In any event, a statistically significant change in the detection signal can be used to facilitate identification of those compounds which are effectors of the phospholipase transducing activity.

In one embodiment, there is provided an assay for identifying a modulator of a phospholipase C activity, comprising the steps of: (i) contacting the cell of claim 1 with a test compound under conditions appropriate for detecting an intracellular signal transduced via the phospholipase-dependent signal pathway; and (ii) measuring a level of signal transduced by the phospholipase-dependent signal pathway in the presence of the test compound. A statistically significant difference in the level of signal in the presence of the test compound, e.g., increase or decrease relative to the absence of test compound, indicates that the test compound is a modulator of the heterologous phospholipase C activity. The intracellular signal can be detected, for example, by expression of a reporter gene operably linked to transcriptional regulatory elements sensitive to the phospholipase-dependent signal pathway. In other embodiments, the intracellular signal is detected by measuring products of the hydrolysis of phosphatidylinositol 4,5-bisphosphate; by measuring Ca^{2+} mobilization; and/or by measuring the activation of a protein kinase such as PKC. In yet other embodiments, the intracellular signal can be detected by measuring a change in phenotype conferred by the heterologous phospholipase, such as a change in temperature sensitivity or NaCl sensitivity.

In a preferred embodiment, the assay makes use the ability of a heterologous PLC to complement a loss-of-function mutation to an endogenous phospholipase activity. This embodiment of the subject assay generally includes the steps of: (i) contacting the cell with a test compound under conditions appropriate for detecting one or both of the phenotypes of the loss-of-function mutation or the complemented phenotype; and (ii) measuring the level or degree of one or both of these phenotype in the presence of the test compound. By comparing this measurement with a similar measurement made in the absence of the test compound or the absence of the heterologous phospholipase C, the ability of the test compound to alter the phenotype, relative to the absence of test compound or phospholipase C, can be used to identify test compounds that are modulators of the heterologous phospholipase C activity.

In yet another embodiment, the assay utilizes a cell which is further engineered with a reporter gene construct which is sensitive to a phospholipase-dependent signal pathway from a heterologous PLC activity. Such assay formats generally include the steps of: (i) contacting the cell with a test compound under conditions appropriate for expression of the reporter gene; (ii) detecting expression of the reporter gene in the presence of the test compound; and (iii) comparing the level of reporter gene expression in the presence of the test compound to the level of reporter gene expression in the absence of the test compound or the absence of the heterologous phospholipase C. A statistically significant difference in the level of reporter gene expression in the presence of the test

compound, relative to the absence of test compound or phospholipase C, indicates that the test compound is a modulator of the heterologous phospholipase C activity.

Still another embodiment of the assay provides a differential screening format for identifying an agent which selectively inhibits a non-mammalian phospholipase C relative to a mammalian phospholipase. Such assay formats can include the steps of: (i) providing a first yeast cell containing a non-mammalian phospholipase gene; (ii) providing a second yeast cell expressing a mammalian phospholipase gene; (iii) contacting each of the first and second yeast cells with a test compound; and (iv) detecting or quantitating the activity of the phospholipases of the first and second cell in the presence of the test compound. A statistically greater decrease in the phospholipase activity of the first cell, relative to the second cell, indicates that the test compound selectively inhibits the non-mammalian phospholipase C. Such differential screening format can be used to identify inhibitors which are selective for phospholipases of mammalian pathogens relative to the host mammalian phospholipase. For instance, the mammalian PLC can be a human PLC, and the non-mammalian PLC can be derived from a human fungal pathogen, e.g. a pathogen which causes a mycosis selected from a group consisting of candidiasis, aspergillosis, mucormycosis, blastomycosis, geotrichosis, cryptococcosis, chromoblastomycosis, penicilliosis, conidiosporosis, nocaidiosis, coccidioidomycosis, histoplasmosis, maduromycosis, rhinosporidosis, monoliasis, para-actinomycosis, and sporotrichosis. In preferred embodiments, the phospholipase of the first cell can be cloned from a human pathogen selected from a group consisting of *Candida albicans*, *Candida stellatoidea*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida pseudotropicalis*, *Candida quillermondii*, *Candida rugosa*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Absidia corymbifera*, *Absidia ramosa*, and *Mucor pusillus*.

In any of the subject drug screening assays described herein, the assay can be carried out, e.g., repeated, for a library of at least 100 different test compounds, though more preferably at least 10^3, 10^4, 10^5, 10^6, or 10^7 different (variegated) compounds. The test compound can be, to illustrate, peptides, nucleic acids, carbohydrates, small organic molecules, natural product extracts, synthetic compounds, or a mixture of combinatorial compounds.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor

Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986).

Detailed Description of the Invention

Phospholipases C (PLC) are a family of enzymes which hydrolyze the sn-3 phosphodiester bond in membrane phospholipids producing diacylglycerol and a phosphorylated polar head group. Mammalian PLC enzymes exhibit specificity for the polar head group which is hydrolyzed, i.e., phosphatidylcholine, phosphatidylinositol, etc. Hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] by specific PLC enzymes generates two second messenger molecules; diacylglycerol, a co-factor required for activation of protein kinase C, and inositol 1,4,5-trisphosphate (IP_3), a soluble second messenger molecule which promotes the release of Ca^{2+} from intracellular stores (Berridge, (1987) *Ann. Rev. Biochem* 56:159-193).

The importance of PLC activation in cell proliferation is evident from the fact that the hydrolysis of PtdIns(4,5)P_2 is one of the early events that follow the interaction of many growth factors and mitogens with their respective receptors. However, the importance of PLC activation is not restricted to proliferation; it is one of the most common transmembrane signaling events elicited by receptors that regulate many other cellular processes, including differentiation, metabolism, secretion, contraction, and sensory perception. Such PLC-mediated transmembrane signaling events include, for example, signaling via receptor tyrosine kinases and G protein coupled receptors.

Moreover, the diacylglycerol released may be further metabolized to free arachidonic acid by sequential actions of diglycerol lipase and monoglycerol lipase. Thus, phospholipases C are not only important enzymes in the generation of second messenger molecules, but may serve an important role in making arachidonic acid available for eicosanoid biosynthesis in select tissues.

The present invention is based on the development of yeast strains which express a functional, heterologous phospholipase and makes available a rapid, effective assay for

screening and identifying pharmaceutically effective compounds that specifically interact with and modulate the activity of the heterologous phospholipase. As described herein, the present invention provides a convenient format for discovering drugs which can be useful to modulate cellular function, as well as to understand the pharmacology of compounds that specifically interact with phospholipases.

Before further description of the invention, certain terms employed in the specification, examples and appended claims are, for convenience, collected here.

"Signal transduction" is the process by which chemical signals are relayed from the cellular environment to cellular targets via the cell membrane, and may occur through one or more mechanisms, such as phosphorylation, activation of ion channels, effector enzyme activation via guanine nucleotide binding protein intermediates, activation of phospholipase, and/or direct activation (or inhibition) of a transcriptional factor.

A "mammalian phospholipase" is a protein which is either identical to an phospholipase occurring naturally in a mammal, or is a mutant which is substantially homologous with such a mammalian phospholipase and more similar in sequence to it than to the yeast phospholipase. Related terms, such as "primate phospholipase", or "human phospholipase", are analogously defined. A mammalian phospholipase is "functionally homologous" to a yeast protein if, either alone, or in concert with other exogenous proteins, or after being modified by a drug, it is able to provide an phospholipase activity within the engineered yeast cell. It is not necessary that it be as efficient as the yeast protein, however, it is desirable that it have at least 10% of the activity of the analogous yeast protein.

An "activator" or "agonist" of a phospholipase is a substance which causes the phospholipase to become more enzymatically active, e.g., elevates the rate at which phospholipid hydroylsis occurs under particular conditions. The mode of action of the activator may be direct, e.g., through binding the phospholipase, or indirect, e.g., through binding another molecule which otherwise interacts with the phospholipase.

Conversely, an "inhibitor or antagonist" of an phospholipase is a substance which causes the enzyme to become less active, and thereby reduces the rate or production of the hydrolytic second messengers to a detectable degree. The reduction may be complete or partial, and due to a direct or an indirect effect.

The term "phospholipase-dependent signal pathway" as used herein refers to a signal transduction pathway including a phospholipase activity as a step in the transduction of the intracellular signal. Such a signal transduction pathway is meant to include components of a pathway whose activity is modulated by a phospholipase, whether their activity is directly modulated by the phospholipase or indirectly modulated by the phospholipase or its second

messengers. This term also includes components of the pathway which are "upstream" of the phospholipase which regulate an activity of the phospholipase.

The term "functional integration" as used herein refers to the ability of an exogenous phospholipase to function in a phospholipase-dependent signal transduction pathway such that a distal effect of a phospholipase activity can be determined, i.e., an activity regulated by the products produced from phospholipase dependent phospholipid hydrolysis. Such distal signaling effects can be observed in yeast expressing either a functional or mutant phospholipase. Another example of functional integration is complementation by a heterologous phospholipase C of one of the pleiotropy of effects seen in phospholipase loss-of-function yeast strains (e.g., reduced growth or NaCl sensitivity). Another assay of functional complementation is the ability of the heterologous phospholipase to modulate gene transcription. Yet another assay of functional integration is the reconstitution of regulated phospholipase activity, i.e., phospholipase activity requiring stimulation, for example phospholipase activity upon receptor-ligand activation or upon activation by a G protein subunit or subunits. This term can also include functional integration of a phospholipase into an endogenous yeast pathway or a chimeric pathway including components not naturally found in yeast cells.

The term "modulation of a phospholipase activity" in its various grammatical forms, as used herein, designates induction and/or potentiation, as well as inhibition of one or more signal transduction pathways involving of a phospholipase. Such modulation may involve effects of a compound directly on a phospholipase, or on a component of a signal transduction pathway involving a phospholipase, for example upstream regulatory elements, or downstream elements, including substrates.

The term "substantially homologous", when used in connection with amino acid sequences, refers to sequences which are substantially identical to or similar in sequence, giving rise to a homology in conformation and thus to similar biological activity. The term is not intended to imply a common evolution of the sequences. Typically, "substantially homologous" sequences are at least 50%, more preferably at least 80%, identical in sequence, at least over any regions known to be involved in the desired activity. Most preferably, no more than five residues, other than at the termini, are different. Preferably, the divergence in sequence, at least in the aforementioned regions, is in the form of "conservative modifications".

The terms "protein", "polypeptide" and "peptide" are used interchangeably herein.

As used herein, "recombinant cells" include any cells that have been modified by the introduction of heterologous DNA. Control cells include cells that are substantially identical to the recombinant cells, but do not express one or more of the proteins encoded by the

heterologous DNA, e.g., do not include or express one or more of the exogenous phospholipase, regulatory protein, test polypeptide, or the reporter gene construct.

As used herein, the terms "heterologous DNA" or "heterologous nucleic acid" is meant to include DNA that does not occur naturally as part of the genome in which it is present or DNA which is found in a location or locations in the genome that differs from that in which it occurs in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell, i.e., is exogenous to the cell. Generally, although not necessarily, such DNA encodes RNA and proteins that are not normally produced by the cell in which it is expressed. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by the term heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes a phospholipase, test polypeptides, regulatory proteins, reporter genes, transcriptional and translational regulatory sequences, or selectable or traceable marker proteins, such as a protein that confers drug resistance.

The terms "recombinant protein", "heterologous protein" and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. That is, the polypeptide is expressed from a heterologous nucleic acid.

A "heterologous phospholipase" refers to a non-yeast phospholipase enzyme, having a phospholipase activity. For example, phospholipase C, in the presence of calcium, can convert phosphatidyl inositol (PI), phosphatidyl inositol 4-monophosphate (PIP) or phosphatidyl inositol (4,5) biphosphate (PIP_2) into at least inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG).

An "endogenous, mutant phospholipase gene" as used herein refers to a yeast phospholipase gene that encodes an endogenous, mutant or impaired yeast phospholipase protein. For example, an endogenous, mutant phospholipase may have a deletion, insertion, point mutation or other mutation that results in a conditional impairment or a constitutive impairment, making them ideal for selection or complementation studies. An example of an endogenous, mutant phospholipase gene is a deletion mutant in the *S. cerevisiae* PLC1 locus (*plc1*). These mutants have several phenotypic differences from wildtype PLC1 strains. *plc1* strains are sensitive to high osmolarity (0.5M NaCl or 1.2M sorbitol) at 30°C and do not grow well in the presence of carbon sources other than glucose (i.e. glycerol/ethanol, raffinose, and galactose). Finally, *plc1* strains have a temperature sensitive phenotype; at

temperatures above 35°C, *plc1* strains do not grow, while at temperatures below 35°C, they grow, albeit slower than wildtype strains.

A "loss of function" mutation is one which leads to a change in the activity of a phospholipase gene. Such a mutation may alter one or more of the coding sequence, the transcription, or the translation of an endogenous yeast phospholipase. Such a mutation will lead to the lack of expression of a yeast phospholipase, or the expression of an inactive yeast phospholipase or a phospholipase of reduced activity.

As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to a transcriptional regulatory sequences. Transcription of the reporter gene is controlled by these sequences. The activity of at least one or more of these control sequences is directly or indirectly regulated by a signal transduction pathway involving a phospholipase, e.g., is directly or indirectly regulated by a second messenger produced by the phospholipase activity. The transcriptional regulatory sequences can include a promoter and other regulatory regions, such as enhancer sequences, that modulate the activity of the promoter, or regulatory sequences that modulate the activity or efficiency of the RNA polymerase that recognizes the promoter, or regulatory sequences that are recognized by effector molecules, including those that are specifically induced upon activation of a phospholipase. For example, modulation of the activity of the promoter may be effected by altering the RNA polymerase binding to the promoter region, or, alternatively, by interfering with initiation of transcription or elongation of the mRNA. Such sequences are herein collectively referred to as transcriptional regulatory elements or sequences. In addition, the construct may include sequences of nucleotides that alter the stability or rate of translation of the resulting mRNA in response to PLC second messages, thereby altering the amount of reporter gene product.

As used herein, a "heterologous regulatory protein" refers to a protein that regulates or is coupled to a heterologous phospholipase. Such a regulatory protein may be upstream or downstream of a phospholipase and is capable of modulating the activity of a phospholipase or being modulated by the phospholipase. A preferred regulatory protein is a GTP binding protein (G protein), which as used herein refers to heterotrimeric G proteins, G protein subunits thereof, e.g. α, β and γ subunits, or a functional heterodimer (e.g. $\beta\gamma$). A regulatory protein as used herein may also be a receptor that modulates a signal transduction pathway involving a phospholipase. Examples of preferred regulatory receptors are those that directly activate a phospholipase, such as the G protein coupled receptors, receptor tyrosine kinases (RTK's), receptors that activate phospholipases via the activation of a non-receptor kinases, such as those that signal via src kinases and the like. Other regulatory proteins can bind PIP_2, the preferred substrate for many PLC isozymes (e.g. profilin, cofilin, gelsolin, and alpha-

actinin) as discussed in Homma and Emori, 1995 and references therein. Regulatory proteins are discussed in further detail below.

As used herein, "cell surface receptor" refers to molecules that occur on the surface of cells, interact with the extracellular environment, and transmit or transduce the information regarding the environment intracellularly in a manner that ultimately modulates transcription of specific promoters, resulting in transcription of specific genes.

As used herein, "extracellular signals" include a molecule or a change in the environment that is transduced intracellularly via cell surface proteins that interact, directly or indirectly, with the signal. An extracellular signal or effector molecule includes any compound or substance that in some manner specifically alters the activity of a cell surface protein. Examples of such signals include, but are not limited to, molecules such as ions, acetylcholine, growth factors, and hormones, that bind to cell surface and/or intracellular receptors and ion channels and modulate the activity of such receptors and channels. Extracellular signals also include as yet unidentified substances that modulate a signal transduction pathway involving a phospholipase, and thereby influence intracellular functions. Such extracellular signals are potential pharmacological agents that may be used to treat specific diseases by modulating phospholipase activity.

I. Overview of Assay

In vitro testing is a preferred methodology in that it permits the design of high-throughput screens: small quantities of large numbers of compounds can be tested in a short period of time and at low expense. Optimally, animals are reserved for the latter stages of compound evaluation and are not used in the discovery phase; the use of whole animals is labor-intensive and extremely expensive. Microorganisms, to a much greater extent than mammalian cells and tissues, can be easily exploited for use in rapid drug screens. Yeast provide a particularly attractive test system; extensive analysis of this organism has revealed the conservation of structure and function of a variety of proteins active in basic cellular processes in both yeast and higher eukaryotes.

As set out above, the present invention relates to recombinant yeast cells expressing a heterologous phospholipase and the methods of using these cells to identify compounds capable of modulating the signal transduction activity of such phospholipases. In general, the subject assay is characterized by the use of recombinant cells which express a heterologous phospholipase protein whose signal transduction activity can be modulated in the recombinant cell so as to generate a detectable signal. As described below, these cells may (optionally) also express one or more of: (i) an expressible recombinant gene encoding an

exogenous test polypeptide from a polypeptide library, (ii) a regulatory protein coupled to a phospholipase activity, and (iii) a reporter construct.

The functional expression of a mammalian phospholipase in yeast provides for the design of inexpensive screens useful in the identification of modulators of the enzyme, the activity which is required for the generation of a central signaling molecule in mammalian cells. Any chemical entity, or combination of chemical entities, whether natural or synthetic, may be screened for the ability to modulate the mammalian phospholipase. These modulators may act directly on the enzyme to alter the activity of the enzyme, e.g., they can competitively, noncompetitively, and/or allosterically potentiate or inhibit the enzyme's activity, or may affect the ability of a regulatory protein to alter phospholipase activity.

In one embodiment, the engineered cell is used to screen for drugs which, like Gα or in some cases Gβγ, can directly activate the phospholipase, or increase the activity of a partially activated phospholipase. In certain embodiments a chimeric Gα or Gβγ may be expressed for this purpose.

In a second embodiment, the engineered cell is used to screen for drugs which inhibit mammalian phospholipase. In this situation, the phospholipase must first be activated. This can be done by engineering the cell to express Gα or Gβγ, as appropriate. Alternatively, the cell may be engineered to co-express both a G protein and a G protein coupled receptor, or any other protein which regulates the activity of the phospholipase, and the receptor stimulated either by externally added ligand or by a co-expressed ligand. In either case, the ligand is a known activator used merely to stimulate activation of the phospholipase, and the drugs are screened for inhibition of this phospholipase.

In a third embodiment, the engineered cell is used to screen for drugs which inhibit or activate phospholipase indirectly, e.g., by their action upon a regulatory protein, for example, a G protein-coupled receptor. The receptor activates the G protein subunits which act on the phospholipase. In this case, a compatible G protein-coupled receptor and a compatible G protein would be provided with the mammalian phospholipase in the same yeast cell.

The ability of particular test compounds to modulate the signal transduction activity of the target phospholipase can be scored for by detecting up or down-regulation of the detection signal. For example, second messenger generation (e.g. phospholipid hydrolysis, Ca^{2+} mobilization or PKC activation) via a phospholipase-dependent signal pathway can be measured directly. Alternatively, the use of a reporter gene can provide a convenient readout. In any event, a statistically significant change in the detection signal can be used to facilitate identification of those compounds which are effectors of the target phospholipase.

By this method, test compounds which modulate phospholipase signaling can be screened. If the test compound does not appear to induce the activity of the phospholipase, the assay may be repeated and modified by the introduction of a step in which the recombinant cell is first contacted with a known activator of the phospholipase, and the test compound can be assayed for its ability to inhibit the activity of the phospholipase, e.g., to identify phospholipase antagonists. Alternatively, the cell can be engineered with an activated phospholipase. In yet other embodiments, test compounds can be screened for members which potentiate the response to a known activator of the phospholipase. In yet another embodiment a phsopholipase or a phospholipase regulatory protein may be constituatively active to facilitate screening for inhibitors of an activated phospholipase. For example, constituatively active forms of the heterotrimeric Gq family members, such as G11 and G16 have been described, such as Gα qQ209L and Gα 16Q212L.

Overexpression these GTPase deficient activated alleles of Gα was found to constitutively elevate basal phospholipase C activity, leading to persistent activation of cJun NH2-terminal kinases (Heasley et al. 1996 *Mol. Cell. Biol.* 16:648; Heasley et al. 1996. *J. Biol. Chem.* 271:349; Qian et al. 1994 *J. Biol. Chem.* 1994. 269:17417). In still a further embodiment a constituatively actived form of a phospholipase activating receptor may be expressed, such as an α 1B-adrenergic receptor which has been mutated to render it constituatively active (Perez et al. 1996. *Mol. Pharmacol.* 49:112) or an altered form of the formylmethionylleucylphenylalanine receptor (Amatruda et al. 1995. *J. Biol. Chem.* 270:28010). In other embodiments the phospholipase itself may be expressed in a constituatively activated form.

In a further embodiment, the subject reagent cells can be used to perform differential screening assays, e.g., which can be used to identify a compound that selectively effects a non-mammalian (e.g. non-human) phospholipase. Such compounds, by selective inhibition, can be used for treating or preventing a pathogen infection (e.g. viral, fungal, bacterial or protozoan) in a mammal (e.g. human). Preferred heterologous phospholipase enzymes for performing differential screening assays against a mammalian phospholipase include phospholipases from viral, fungal or protozoan pathogens. Examples of fungal pathogens include fungi that cause Candidiasis, Aspergillosis, Mucormycosis, Blastomycosis, Geotrichosis, Cryptococcosis, Chromoblastomycosis, Coccidioidomycosis, Conidiosporosis, Histoplasmosis, Maduromycosis, Rhinosporidosis, Nocaidiosis, Para-actinomycosis, Penicilliosis, Monoliasis, and Sporotrichosis.

In a further embodiment of a differential screen, the ability of a compound to modulate the activity of one phospholipase and not another phospholipase may be measured. For example, compounds which are agonists or antagonists of PLCβ and not PLCγ or are

capable of modulating PLCβ2 and not PLCβ3 may be selected. In yet another embodiment compounds which are capable of modulating a heterologous phospholipase while not affecting another member of a signal transduction protein which may or may not be a member of a phospholipase pathway, such as PKC may be tested.

In developing the recombinant cells assays, it was recognized that a frequent result of the activation of a phospholipase signaling pathway was the transcriptional activation or inactivation of specific genes, i.e., the activation of a phospholipase often ultimately leads to a rapid and detectable change in the transcription or translation of a gene. Thus, transcription of genes controlled by phospholipase-responsive transcriptional elements often reflects the activity of the surface protein by virtue of transduction of an intracellular signal.

To illustrate, the intracellular signal that is transduced can be initiated by the specific interaction of an extracellular signal, particularly a ligand, with a cell surface receptor which is coupled to the phospholipase. This interaction sets in motion a cascade of intracellular events, the ultimate consequence of which is a rapid and detectable change in the transcription or translation of a gene. By selecting transcriptional regulatory sequences that are responsive to the phospholipase-dependent intracellular signals and operatively linking the selected promoters to reporter genes, whose transcription, translation or ultimate activity is readily detectable and measurable, the transcription based assay provides a rapid indication of whether a specific test compound influences intracellular transduction. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as agonists or antagonists of phospholipase activities.

Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation and are thus ideal for assays in which a heterologous phospholipase is functionally integrated into a signal transduction pathway. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on 2nd messengers generated by the phospholipase activity. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the signal transduction activity of the target phospholipase, with the level of expression of the reporter gene providing the phospholipase-dependent detection signal. The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, specific mRNA expression may be detected using Northern blots or specific protein product may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound or it may be compared with the amount of transcription in a

substantially identical cell that lacks the target phospholipase. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the activity of the target phospholipase.

As described in further detail below, in preferred embodiments the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence.

In other preferred embodiments, the reporter or marker gene provides a selective growth advantage, e.g., the reporter gene may enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug.

In other embodiments, second messenger generation can be measured directly in the detection step, such as mobilization of intracellular calcium or phospholipid metabolism are quantitated, for instance, the products of phospholipid hydrolysis IP3 or DAG could be measured For example, one of the downstream effects of PLC activation in mammalian cells is Ca^{++} mobilization; because the teachings of the present invention demonstrate that a mammalian phospholipase can be functionally integrated into a yeast cell, it is likely that this effect may be expected in yeast cells expressing functional, recombinant PLCs. Similarly, as mammalian PLC is known to modulate PKC via its second messenger products (including DAG) functional expression of mammalian PLCs in yeast may be expected to couple to other signal pathway effectors including IP3-receptor, CaM kinase, calmodulin, PKA and PKC, or other proteins which interact with the phospholipases.

II. Host Cells

The host yeast cell of the present invention may be of any species which is cultivable and in which an exogenous phospholipase can be modulated. Suitable species include *Kluyverei lactis, Schizosaccharomyces pombe*, and *Ustilaqo maydis; Saccharomyces cerevisiae* is preferred. Other yeast which can be used in practicing the present invention are *Neurospora crassa, Aspergillus niger, Aspergillus nidulans, Pichia pastoris, Candida tropicalis, and Hansenula polymorpha.* The term "yeast", as used herein, includes not only yeast in a strictly taxonomic sense, i.e., unicellular organisms, but also yeast-like multicellular fungi or filamentous fungi.

The choice of appropriate host cell will also be influenced by the choice of detection signal. For instance, reporter constructs, as described below, can provide a selectable or screenable trait upon transcriptional activation (or inactivation) in response to a signal transduction pathway coupled to the target receptor The reporter gene may be an

unmodified gene already in the host cell pathway, such as the genes responsible for growth arrest in yeast. It may be a host cell gene that has been operably linked to a "phospholipase-responsive" promoter. Alternatively, it may be a heterologous gene that has been so linked. Suitable genes and promoters are discussed below. Accordingly, it will be understood that to achieve selection or screening, the host cell must have an appropriate phenotype. For example, introducing a pheromone-responsive chimeric HIS3 gene into a yeast that has a wild-type HIS3 gene would frustrate genetic selection. Thus, to achieve nutritional selection, an auxotrophic strain will be desired.

"Inactivation", with respect to genes of the host cell, means that production of a functional gene product is prevented or inhibited. Inactivation may be achieved by deletion of the gene, mutation of the promoter so that expression does not occur, or mutation of the coding sequence so that the gene product is inactive. Inactivation may be partial or total.

Accordingly, in certain embodiments it may be desirable to disrupt an endogenous yeast phospholipase to facilitate screening for an activity of a heterologous phospholipase. The field of yeast genetics is advanced and techniques for disrupting yeast genes are known in the art. For example, transplacement may be used to replace the endogenous yeast phospholipase gene with a modified form, as described by Flick and Thorner in art. In the case of yeast PLC1, a *plc*Δ1::HIS3 mutation can be created by replacing the internal *Pvu*II fragment of PLC1 (nucleotides +1069 to +1482) with *HIS3*, as follows. The 3.7-kb *Eco* RV fragment containing most of *PLC1* can be inserted into *Eco*RV-digested YCp50 to (Rose et al. 1987. *Gene*. 60:237) to yield pJF10, which can be cleaved with *Pvu*II and ligated with the 2.1-kb PvuII fragment of pJJ215 that contains *HIS3* (Jones and Prakash. 1990 *Yeast* 6:363). In the resulting plasmid (pJF18), *HIS3* has replaced residues 357 to 494 of Plc1p. The *plc1Δ1::HIS3* allele can be introduced into yeast cells by DNA mediated transformation (Rothstein. 1983. *Methods Enzymol.* 101:202) of two different *his3/his3* diploid strains, YPH501 (Sikorski and Heiter. 1989 *Genetics* 122:19) and W303 (Thomas and Rothstein 1989. Cell 56:619), using *Eco*RV-digested pJF18 and selecting for histidine prototrophy.

A *plc1Δ2*::LEU2 mutation can be created by replacing the *Hpa*I-*Cla*I segment of *PLC1* (nucleotides +277 to +2333) with *LEU2*, as follows. pJF58, which consists of a 4.3-kb *Hind*III fragment containing *PLC1* inserted in pUC19 can be digested with *Hpa*I and *Cla*I and incubated with the Klenow fragment of *E. coli* DNA polymerase I. The resulting 5.4-kb linearized vector can be gel purified and ligated with a 2.2 kb *Sal*I-*Xho*I fragment containing *LEU2* that can be excised from pJJ283 (Jones and Prakash, *supra*) and converted to flush ends by treatment with Klenow enzyme, yielding pJF96. The *plc1*Δ2::*LEU2* allele, which is almost a complete deletion of the *PLC1* gene, can be introduced into *S. cerevisiae* by DNA-

mediated transformation of the *leu2/leu2* diploid strain, YPH501, using *Hind*III-digested pJF96 and selecting for leucine prototrophy.

Transplacement of the resident *PLC1* locus by the mutant allele on only one homolog in each of the diploid transformants can be confirmed by restriction enzyme digestion and Southern blot hybridization analysis (Sambrook et al. 1989. Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The phenotype of asci from sporulated cultures of heterozygous *plc1Δ/PLC1* diploids may be determined as in the art.

Ideally, any mutants generated will have a selectable phenotype, such as, for example, cessation of growth at 37°C, or failure to grow on a particular carbon source, will exhibit sensitivity to hypertonic stress (e.g., to salt or sorbitol), or will fail to grow on medium lacking a nitrogen source. In preferred embodiments a yeast cell expressing an endogenous mutant PLC will be capable of growth under one set of experimental conditions and will fail to grow or exhibit growth impairment under a second set of experimental conditions, i.e., would be conditionally lethal.

"Complementation", with respect to genes of the host cell, means that at least partial function of inactivated gene of the host cell is supplied by an exogenous nucleic acid. For instance, yeast cells can be "mammalianized", and even "humanized", by complementation of receptor and signal transduction proteins with mammalian homologs. To illustrate, inactivation of a yeast phospholipase gene can be complemented, as described in the appended examples, by expression of a human phospholipase gene.

The complementation of the *plc1Δ* ts phenotype observed with human PLC-β2 and Gαq family members can be used in a screening format to identify a variety of agonists and antagonists directed to these functionally interacting proteins. PLC-β agonists can be identified in a screen for compounds that stimulate growth of *plc1Δ* yeast strains expressing PLC-β2 but not expressing any Gαq family Gα-subunit under non-permissive growth conditions. *plc1Δ* strains expressing wildtype yeast PLC1 could serve as an appropriate counterscreening strain. PLC-β antagonists could be screened for in two formats. In the first format, plc1Δ yeast strains whose growth under non-permissive conditions is not dependent upon co-expression of Gαq subunits (i.e. strains expressing mammalian PLC-β1, -β2, or -β3 on high copy plasmids) could be screened for compounds that block growth at 37°C or on 0.5M NaCl at 30°C but not at 30°C. An additional counterscreen for this format would be to screen for compounds that block growth of the *plc1Δ* strains but not of PLC1 strains under non-permissive conditions. This counterscreen would serve to distinguish between compounds that act on fungal and mammalian PLC isoforms as opposed to those that act on one or the other or both. In the second format, *plc1Δ* yeast strains whose growth under non-

permissive conditions is dependent upon co-expression of Gαq subunits (i.e. strains expressing PLC-β2 on a low copy plasmid) could be screened for loss of complementation when contacted with compounds. This screen could in theory detect compounds that act either by inhibiting PLC-isoforms directly or by blocking the interaction between PLC-β isoforms and Gαq subunits or by blocking the activation of Gαq subunits. As a final example of the use of functional complementation in screening, it may be possible to construct yeast strains in which the activation or inhibition of signaling components downstream of PLC-β can be scored appropriately configured strains. For example, strains expressing both mammalian PLC-β isoforms and PKC may be used to screen for compounds that modulate the activity of either PLC-β isoforms, PKC isoforms, or both.

Other complementations for use in the subject assay can be constructed without any undue experimentation. Indeed, numerous examples of yeast genetic complementation with mammalian signal transduction proteins have been described in the art. For example, the following table includes a list of some of the human genes which have been found to complement of yeast genes (see also Tuendreich et al. 1994. *Hum. Mol. Gen.* 3:1509)

Table 2. Sequence similarity between Human cDNAs that complement *S. cerevisiae* mutants and their correlate yeast proteins

Human Gene	GenBank Accession #	BLASTX P-value	Yeast Mutant	SwissProt Accession #	Reference
ARF5	M57567	1.0E-91	arf2	P19146	Lee FJ. et al. (1992)
ARF5	M57567	1.4e-91	arf1	P11076	Lee FJ. et al. (1992)
ARF6	M57763	3.1e-85	arf2	P19146	Lee FJ. et al. (1992)
ARF6	M57763	4.0e-84	arf1	P11076	Lee FJ. et al. (1992)
Calmodulin	D45887	7.7e-63	cmd1	P06787	Davis TN. et al. (1989)
CBS	L14577	9.4e-116	cys4	P32582	Kruger WD. et al. (1994)
CCND1	M64349	Not Detected	cln1	P20437	Xiong et al. (1991)
CCND1	M64349	Not Detected	cln2	P20438	Xiong et al. (1991)
CCND1	M64349	Not Detected	cln3	P13365	Xiong et al. (1991)
CDC2	X05360	2.0e-130	cdc28	P00546	Ninomiya-Teuji et al. (1991)
CDC34	L22005	5.1e-38	cdc34	P14682	Plon et al. (1993)
CDK2	X61622	1.3e-137	cdc28	P00546	Elledge et al. (1991)

CDK3	X66357	1.3e-137	cdc28	P00546	Meyerson et al. (1992)
CHK	D10704	2.8e-20	ckil	P20485	Hosaka et al. (1992)
CKS1	X54941	8.7e-23	cks1	P20486	Richardson HE et al. (1990)
CKS2	X54942	6.9e-14	cks1	P20586	Richardson et al. (1990)
CSBP1	L32563	7.2e-117	hog1	P32485	Kumar et al. (1995)
CYC1	X06994	1.2e-98	cyc1	P00044	Tanaka Y. et al. (1988)
Cyclin E	M73812	Not Detected	cln1	P20437	Lew DJ. et al. (1991)
Cyclin E	M73812	Not Detected	cln2	P20438	Lew DJ. et al. (1993)
Cyclin E	M73812	Not Detected	cln3	P13365	Lew DJ. et al. (1991)
DHODH	M94065	1.4e-5	ural	P28272	Minet M. et al. (1992)
eIF-2 alpha	M85294	8.3e-24	gcn2	P15442	Dever TE et al. (1993)
eIF-5A (eIF-4D)	M23419	1.6e-69	tif51B	P19211	Schwelberger HG. et al. (1993)
eIF-5A (eIF-4D)	M23419	1.7e-70	tif51A	P23301	Schwelberger HG. et al. (1993)
FLRN	M59849	1.3e-120	nop1	P15646	Jansen RP. et al. (1992)
G25K	M35543	2.3e-108	cdc42	P19073	Munemitsu S. et al. (1990)
G25K	M35543	Not Detected	cdc24	P11433	Munemitsu S. et al. (1990)
GALK2	M84443	1.5e-64	gal1	P04385	Lee PT. et al. (1993)
GALT	M96264	2.5e-85	gal7	P08431	Fridovich-Keil JL. et al. (1993)
GART	X54199	2.0e-248	ade5, 7	P07244	Schild D. et al. (1990)
GART	X54199	4.8e-12	ade8	P04161	Schild D. et al. (1990)
GBE1	L07956	7.0e-290	glc3	P32775	Thon VJ et al. (1993)
GOS8	L13463	1.4e-1	get2	P11972	Siderovski DP et al. (1996)
GSPT1	X17644	6.3e-165	get2	P05453	Hoshino S. et al. (1989)
HAP2	M59079	2.2e-23	hap2	P06774	Becker DM et al. (1991)
hFKB12	M80199	9.0e-43	rbp1	P20081	Koltin Y. et al. (1991)
H-GRF55	S62035	6.2e-29	cdc25	P04821	Schweighoffer F. et al. (1991)
HHR6A	M74524	1.2e-73	rad6	P06104	Koken MH et al. (1991)
HHR6B	M74525	6.7e-74	rad6	P06104	Koken MH. et al. (1991)
HMGCR	M11058	1.0e-145	hmg2	P12684	Basson ME. et al. (1988)

HMGCR	M11058	4.6e-160	hmg1	P13683	Basson ME. et al. (1988)
hRPB7.0	Z47727	3.9e-13	rpc10	P40422	Shpakovski GV et al. (1995)
hRPB7.6	Z47728	3.9e-13	rpb10	P22139	Shpakovski GV et al. (1995)
hRPB17	Z49199	1.0e-23	rpb8	P20436	Shpakovski GV et al. (1995)
hRPB14.4	Z27113	2.8e-40	rpo26	P20435	Shpakovski GV et al. (1995)
LIG1	M36067	2.3e-168	cdc9	P04819	Barnes DE. et al. (1990)
MGMT	M29971	1.9e-8	mgt-1	P26188	Xiac W. et al. (1995)
MSS1	D11094	8.4e-211	cim5	P33299	Ghislain M. et al. (1993)
MTTF1	X64269	Not Detected	abf2	002486	Parisi MA. et al. (1993)
NF1	M89914	1.5e-38	ira2	P19158	Ballester R. et al. (1990)
NMT	M86707	9.7e-122	nmt1	P14783	Duronio RJ. et al. (1992)
OAT	M12267	9.7e-133	car2	P07991	Dougherty KM. et al. (1993)
Oxidoreductase	S90469	6.7e-83	ncpr1	P16603	Eugster HP. et al. (1002
PDE4A	M37744	Not Detected	pde1	P22434	McHale et al. (1991)
PDE4A	M37744	6.4e-15	pde2	P06776	McHale et al. (1991)
PDE7	L12052	Not Detected	pde1	P22434	Michaeli T. et al. (1993)
PFKM	M26066	1.9e-192	pfk	P16861	Heinisch JJ. et al. (1993)
PYCR1	M77836	4.9e-21	pro3	P32263	Dougherty KH. et al. (1993)
RCC1	D00591	1.4e-13	srm1 (prp20)	P34760	Clark KL. et al. (1991)
SEC13r	L09260	2.1e-116	sec13	Q04491	Shaywitz DA. et al. (1995)
snRMP D1	J03798	1.1e-20	smd1	Q03260	Rymond BC. et al. (1993)
SPT4	T50643	6.0e-19	spt4	P32914	Hartzog G. et al. (1996, submitted)
TOP1	J03250	6.1e-195	top1	P04786	Bjornsti MA. et al. (1989)
TYMK	L16991	4.1e-47	cdc8	P06776	Su JY. et al. (1991)
UBCEP1	M24507	4.5e-46	ubi3	P04839	Monia BP et al. (1990)
VDAC1	L06132	9.8e-15	por1	P04840	Blachly-Dyson E. et al. (1993)
VDAC2	L06328	2.8e-21	por1	P04840	Blachly-Dyson E. et al. (1993)
XPD	X52221	2.9e-293	rad3	P06839	Guzder SN. et al. (1995)

Methods for introducing heterologous DNA into eukaryotic cells are well known in the art and any such method may be used. In addition, cDNA encoding various phospholipases and other of the recombinant proteins described herein are known to those of skill in the art, or may be cloned by any method known to those of skill in the art. Generally, ligating a polynucleotide coding sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well-known proteins, including sequences encoding exogenous receptor and peptide libraries. Similar procedures, or modifications thereof, can be employed to prepare recombinant reagent cells of the present invention by tissue-culture technology in accord with the subject invention.

In general, it will be desirable that the vector be capable of replication in the host cell. It may be a DNA which is integrated into the host genome, and thereafter is replicated as a part of the chromosomal DNA, or it may be DNA which replicates autonomously, as in the case of a plasmid. In the latter case, the vector will include an origin of replication which is functional in the host. In the case of an integrating vector, the vector may include sequences which facilitate integration, e.g., sequences homologous to host sequences, or encoding integrases.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used.

Moreover, it will be understood that the expression of a gene in a yeast cell requires a promoter which is functional in yeast. Suitable promoters include the promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255, 2073 (1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Req.* 7, 149 (1968); and Holland et al. *Biochemistry* 17, 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phospho-fructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phospho-glucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., EPO Publn. No. 73,657. Other promoters, which have the additional advantage of transcription

controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization. Finally, promoters that are active in only one of the two haploid mating types may be appropriate in certain circumstances. Among these haploid-specific promoters, the pheromone promoters MFa1 and MFα1 are of particular interest.

III. Phospholipases

As set out above, one aspect of the present invention encompasses the functional expression of a heterologous phospholipase in a yeast cell. A preferred phospholipase is phospholipase C. Preferred PLCs for carrying out the subject screening assays include any isotype of any of the four classes of phospholipases C (e.g. α, β_{1-4}, γ 1&2 ,or δ_{1-3}). The phospholipases may be recombinant forms of vertebrate (e.g. mammalian, such as bovine, porcine, caprine, laprine, feline, canine, rodent, human or non-human primate), invertebrate (e.g. insect), bacterial, non-bacterial or viral phospholipases. Preferred phospholipases of the present invention are mammalian, with particularly preferred phospholipases being of human origin. Several human PLCs have also been cloned. For example, PLC (Genbank Accession number X14034), PLC1 (Genbank Accession number M37238), PLCβ2 (Genbank Accession number M95678; see also Park et al. *J. Biol. Chem.* 1992. 267:16048) and PLCβ3 (Genbank Accession number Z37566), see also Ohta, Matsui, Nazawa, Lagercrantz et al. (*Genomics* (1995) 20:467:472); PLCβ4 (Genbank Accession number L41349) see also Alvarez et al. (1995) *Genomics* 29:53); PLCγ1 (Genbank Accession number U09117) see also Cheng et al (1995) *J. Biol. Chem* 270:5495); PLCγ (Genbank Accession number M34667) see also Burgess et al. (1990) *Mol Cell Biol.* 10:4770) and PLCα (Genbank accession number D16234) see also Hirano et al. (1994) *Biochem Biophys Res Commun* 204:375.

Moreover, the heterologous phospholipase need not be a naturally occurring protein, rather, it may be a mutant form of a phospholipase. Preferably, the mutant is substantially homologous to a naturally occurring phospholipase, or a mutant known to be functional.

Moreover, the structural gene encoding the phospholipase may be the wild-type mammalian gene, or a modified gene. "Silent" modifications may be made to improve expression, by, e.g., (1) eliminating secondary structures in the corresponding mRNA, or (2) substituting codons preferred by yeast for codons that are not so preferred, or to facilitate cloning, e.g., by introducing, deleting or modifying restriction sites. The gene may also be modified so that a mutant phospholipase is encoded.

Analysis of yeast codon usage indicates that there exists a preferred codon set consisting of the most abundant isoaccepting tRNAs present in yeast and that this preferred set (25 out of the 61 possible coding triplets) is the same for all yeast proteins (Bennetzen and Hall (1981) J. Biol. Chem. 257, 3026-3031). The rapid translation rate required for abundant proteins is believed to provide the selective pressure for the existence of the preferred set of codons. As the extent of biased codon usage in specific genes correlates directly with the level of gene expression (Hoekma et al. (1987) Mol. Cell. Biol. 7, 2914-2924), experimental strategies aimed at the expression of heterologous genes in yeast exploit the codon bias that has been described for that organism (Sharp et al. (1986) Nuc. Acids Res. 14, 5125-5143).

For instance, as described in details in the Exemplification, the amino terminal seven residues of PLC-β1 and the amino terminal 21 residues of human PLC-β2 can be mutated using synthetic oligonucleotides to effect these changes. The nucleotide sequence of each construct may be subsequently verified by dideoxynucleotide sequencing methods on both DNA strands. Other desirable, conservative amino acid substitutions not specifically cited here may be made to the phsopholipase sequence without any diminishment of wild type protein activity.

IV. Other Regulatory Proteins

In certain embodiments, the yeast cells of the present invention can be further engineered to express one or more regulatory proteins which regulate an exogenous phospholipase. Preferred regulatory proteins are G-protein coupled receptors, G proteins, receptor tyrosine kinases, and/or non-receptor kinases. Examples of other regulatory proteins are proteins which bind to phospholipase substrates and influence the activity of a phospholipase, such as profillin. Phospholipase regulatory proteins are described in detail below.

A. G Protein-Coupled Receptors

One family of signal transduction cascades found in eukaryotic cells utilizes heterotrimeric "G proteins." Transduction of growth signals by G protein-coupled receptors has been demonstrated to include phospholipase-dependent pathways. For example, β-type PLC isoforms are activated by the heterotrimeric G protein subfamily Gq. The α-subunits of $G_{q/11/16}$, to further illustrate, specifically regulate PLC-β1 and PLC-β3, and the β/γ-subunits of the Gi subfamily interact with PLC-β2. Agonist interaction with specific G protein-coupled receptors causes the dissociation of Gq proteins into Gα and G β/γ subunits and the exchange of GDP bound to Gα for GTP. The resulting GTP-bound Gα subunit then activates certain PLC isoforms, such as PLCβ's, by binding to the enzyme. G protein signaling systems include three components: a receptor , a GTP-binding protein (G protein), and an intracellular target protein.

In their resting state, the G proteins, which consist of alpha (α), beta (β) and gamma (γ) subunits, are complexed with the nucleotide guanosine diphosphate (GDP) and are in contact with receptors. When a ligand engages the receptor, the receptor changes conformation and this alters its interaction with the G protein. This spurs the α subunit to release GDP, and the more abundant nucleotide guanosine triphosphate (GTP), replaces it, thus activating the G protein. The G protein then dissociates to separate the α subunit from the still complexed β and γ subunits. The activated state lasts until the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the α subunit. Either the Gα subunit, or the Gβγ complex, depending on the pathway, interacts with an effector such as a phospholipase. In the instance of phospholipases, the enzyme in turn converts an inactive precursor molecule into an active "second messenger," which may diffuse through the cytoplasm, triggering a metabolic cascade. Over time, the Gα converts the GTP to GDP, thereby inactivating itself. The inactivated Gα may then reassociate with the Gβγ complex.

Hundreds, if not thousands, of receptors transduce signals through heterotrimeric G proteins, of which at least 17 distinct forms have been isolated. Although the greatest variability has been seen in the α subunit, several different β and γ structures have been reported. There are, additionally, several different G protein-dependent effectors.

Most G protein coupled receptors are comprised of a single protein chain that is threaded through the plasma membrane seven times. Such receptors are often referred to as seven transmembrane receptors (STRs). The following is a partial list of seven transmembrane domain receptors that have been reported in the literature to be coupled to phosphoinositide metabolism in cells and accordingly suitable for generating the subject reagent cell: an α1A-adrenergic receptor, an α1B-adrenergic receptor, an α1C-adrenergic receptor, an M_1 ACh receptor, an M_3 ACh receptor, an M_5 ACh receptor, a D_2 dopamine receptor, a D_3 dopamine receptor, an A1 adenosine receptor, a 5HT1-like receptor, a 5HT1d-like receptor, a 5HT1d beta receptor, a substance K (neurokinin A) receptor, a f-Met-Leu-Phe (FMLP) receptor, an angiotensin II type 1 receptor, a *mas* proto-oncogene receptor, an endothelin ETA receptor, an endothelin ETB receptor, a thrombin receptor, a growth hormone-releasing hormone (GHRH) receptor, a vasoactive intestinal peptide receptor, an oxytocin receptor, a SST3 receptor, an Lutinizing hormone/chorionic gonadotropin (LH/CG) receptor, a thromboxane A2 receptor, a platelet-activating factor (PAF) receptor, a C5a anaphylatoxin receptor, an Interleukin 8 (IL-8), IL-8A receptor, an IL-8B receptor, a mip-1/RANTES receptor, a metabotropic glutamate mGlu1-5 receptor, an ATP receptor, an amyloid protein precursor receptor, a bradykinin receptor, a gonadotropin-releasing hormone receptor, a cholecystokinin receptor, an antidiuretic hormone receptor, an adrenocorticotropic hormone II receptor, LTB4 receptor, LTD4 receptor, tachykinin receptor, thyrotropin releasing hormone receptor, vasopressin receptor and oxytocin receptor Cloning references for exemplary G protein coupled recepotors are provided in Table 1.

TABLE 1 HUMAN G PROTEIN-COUPLED SEVEN TRANSMEMBRANE RECEPTORS: REFERENCES FOR CLONING

RECEPTOR	REFERENCE
α_{1A}-adrenergic receptor	Bruno et al. (1991)
α_{1B}-adrenergic receptor	Ramarao et al. (1992)
muscarinic receptors: M_1 AChR, M2 AChR, M_3 AChR,	Bonner et al (1987) Peralta et al. (1987)
M5 AChR	Bonner et al. (1988)
D_2 dopamine	Grandy et al. (1989)1

D_3 dopamine	Sokoloff et al. (1990)
Al adenosine	Libert et al. (1992)
5-HT1a	Kobilka et al. (1987) Fargin et al. (1988)
5-HT1b	Hamblin et al. (1992) Mochizuki et al. (1992)
5HT1-like	Levy et al. (1992a)
5-HT1d	Levy et al. (1992b)
5HT1d-like	Hambln and Metcalf (1991)
5HT1d beta	Demchyshyn et al. (1992)
substance K (neurokinin A)	Gerard, et al. (1991); Takeda et al. (1991)
f-Met-Leu-Phe	Boulay et al. (1991) Murphy & McDermott (1991) DeNardin et al. (1992)
angiotensin II type 1	Furuta et al. (1992)
mas proto-oncogene	Young et al. (1986)
endothelin ETA	Hayzer et al. (1992) Hosoda et al. (1991)
endothelin ETB	Nakamuta et al. (1991) Ogawa et al. (1991)
thrombin	Vu et al. (1991)
growth hormone-releasing hormone (GHRH)	Mayo (1992)
vasoactive intestinal peptide (VIP)	Sreedharan et al. (1991)
oxytocin	Kimura et al. (1992)
somatostatin SSTR3	Yamada et al. (1992a) Yamada et al. (1992b)
Leutenizing hormone/chorionic gonadotrophin	Minegish et al. (1990)
thromboxane A2	Hirata et al. (1991)
platelet-activating factor (PAF)	Kunz et al. (1992)
C5a anaphylatoxin	Boulay et al. (1991) Gerard and Gerard (1991)
IL-8RA	Holmes et al. (1991)
IL-8RB	Murphy and Tiffany (1991)
MIP-1/RANTES	Neote et al. (1993) Murphy et al., in press

metabotropic glutamate mGluR1-6	Tanabe et al. (1992)
ATP	Julius, David (unpub.)
amyloid protein precursor	Kang et al. (1987) Mita et al. (1988) Lemaire et al. (1989)
bradykinin	Hess et al. (1992)
gonadotropin-releasing hormone	Chi et al. (1993)
cholecystokinin	Pisegna et al. (1992)
antidiuretic hormone receptor	Birnbaumer et al. (1992)
adrenocorticotropic hormone II	Mountjoy et al. (1992)

Specific human G protein-coupled receptors for which genes have been isolated and for which expression vectors could be constructed include those listed herein and others known in the art. Thus, the gene would be operably linked to a promoter functional in the cell to be engineered and to a signal sequence that also functions in the cell. For example in the case of yeast, suitable promoters include those derived from Ste2, Ste3 and gal1 genes. Suitable signal sequences include those of Ste2, Ste3 and of other genes which encode proteins secreted by yeast cells. As appropriate, the yeast genome is preferably modified so that it is unable to produce the yeast analog of the recombinant receptor in functional form.

B. G protein subunits

PLC-β enzymes are activated in response to ligand binding to G-protein coupled receptors by hormones, neurotransmitters, small molecules and other agonists. This occurs via a pertussis toxin insensitive mechanism mediated by members of the Gq family of Gα subunits as well as via a pertussis toxin sensitive mechanism mediated by G-protein complexes containing Gα subunits of the Gi or Go family. With respect to activation of PLC-β enzymes by Gq family members, all members of the Gq family (q, 11, 14, 15, and 16) have been demonstrated both *in vitro* and in cell culture systems to directly stimulate the catalytic activity of PLC-β1, -β2, and -β3 and -β4 enzymes (A.J. Morris et al 1990, and references therein; Sternweis and Smrka, 1992; Wu et al. 1992; and Lee et al. 1992). Members of other Gα subunit families (Gi, Go, Gt, Gz), on the other hand, do not directly stimulate PLC-β activity. The observation that in some cell types stimulation of PLC-β enzymes is sensitive to pertussis toxin treatment is now understood to be due to the direct effect of Gβγ subunits on PLC-β isozymes (summarized in Boyer et al. 1994; and Clapham and Neer, 1993). Recent work from a number of laboratories has demonstrated that PLC-β isoforms (in particular PLC-β2 and -β3) are sensitive to stimulation directly by Gβγ subunits.

Thus, Gβγ subunits derived from Gαi or GαO-containing heterotrimers could contribute to the pertussis toxin sensitivity of PLC-β stimulation observed in whole cell systems in the past. Certain of the regions of PLC-β isozymes that are required for activation by Gα or Gβγ subunits have been characterized. For instance, deletion mutations have shown that residues at the carboxyl terminus of PLC-β1 (Wu et al, 1993) are crucial for interaction with Gα subunits, while residues at the amino terminus appear to be essential for interaction with Gβγ subunits, perhaps through binding of Gβγ subunits to a presumptive pleckstrin homology present in the amino terminal 150 residues of PLC-β2 (Inglese et al. 1995, and references therein).

In certain embodiments of the invention it may be desirable to express both a phospholipase and one or more exogenous G protein subunits, for example, a G protein α subunit. If the exogenous G α subunit is not adequately coupled to the endogenous yeast Gβγ or G protein coupled receptor, the Gα subunit may be modified to improve coupling. These modifications often will take the form of mutations which, relative to interaction with the receptor or Gβγ complex, increase the resemblance of the Gα subunit to the yeast Gα while decreasing its resemblance to the receptor-associated Gα. For example, a residue may be changed so as to become identical to the corresponding yeast Gα residue, or to at least belong to the same exchange group of that residue. After modification, the modified Gα subunit might or might not be "substantially homologous" to the foreign and/or the yeast Gα subunit.

The modifications are preferably concentrated in regions of the Gα which are likely to be involved in one or both of receptor binding and Gβγ binding. In some embodiments, the modifications will take the form of replacing one or more segments of the receptor-associated Gα with the corresponding yeast Gα segment(s), thereby forming a chimeric Gα subunit. (For the purpose of the appended claims, the term "segment" refers to three or more consecutive amino acids.) In other embodiments, point mutations may be sufficient.

This chimeric Gα subunit will interact with the endogenous receptor and the yeast Gβγ complex, thereby permitting signal transduction to occur via a pathway which includes the heterologous phospholipase. While use of the endogenous yeast Gβγ is preferred, if a foreign or chimeric Gβγ capable of transducing the signal to the recombinant phospholipase may be used instead.

C. Tyrosine Kinases

Certain phospholipase, such as PLC-γ enzymes, are activated directly in response to phosphorylation of key tyrosine residues by both receptor and non-receptor tyrosine kinases

(Summarized in Cook and Wakelam, 1992b). Examples of receptors which mediate PLCγ activation include those for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), nerve growth factor (NGF), and erbB2 which have intrinsic tyriosine kinase activity (Kumjian et al. (1991) *J. Biol. Chem.* 266:3973; Margolsis et al. 1989. *Cell* 57:1101; Meisenhelder et al. 1989. *Cell* 57:1109; Mohammadi et al. 1991. *Mol. Cell. Biol.* 11:5068; Stephens et al. 1994. *Neuron* 12:691). Other receptor systems which are devoid of intrinsic enzymatic activity are also linked to PLC. These include receptors for a range of cytokines (Boulton et al. 1994. *J. Biol. Chem.* 269:11648), erythropoietin (Res et al. 1994. *J. Biol. Chem.* 269:19633), and thrombin (Guinebault et al. 1993. *Biochem J.* 292:851), as well as multisubunit immune recognition receptors (MIRRs) such as membrane immunoglobulin M and CD40 in B lymphocytes (Coggeshall, et al. 1992. *Proc. Natl. Acad. Sci. USA* 89:5660; Ren et al. 1994. *J. Exp. Med* 179:673), the T-cell antigen receptor (Park et al. 1991. *Proc. Natl. Acad. Sci. USA* 88:5453; Weiss and Littman. 1994. *Cell* 76:253), the high-affinity immunoglobulin E receptor in basophilic leukemia cells (Li et al. 1992. *Mol. Cell. Biol.* 12:3176; Park et al. 1991. *J. Biol. Chem.* 266:24237), and the immunoglobulin G receptor in monocytic cells (Liao et al. 1993. Biochem. Biophys. Res. Commun. 191:1028). When the receptor has no intrinsic tyrosine kinase activity, non-receptor protein tyrosine kinases coupled to the receptor mediate the response, such as members of the Src and Syk/ZAP70 family, including lyn, syk and fyn (Liao et al. *supra*; Rhee et al. 1992. *Adv. Second Messenger Phosphoprotein Res.* 26:35-61; Weiss and Littman, *supra*).

PLC-γ isozymes bind to specific autophosphorylated tyrosine residues within the kinase domains of these PTKs via the SH2 domains present in the spacer region between the PLC X and Y regions and the PLCs themselves become substrates for tyrosine phosphorylation. Phosphorylation of specific tyrosine residues (Tyr783 and Tyr1254 on PLC-γ1 and Tyr753 and Tyr759 on PLC-γ2) by the receptor associated tyrosine kinase activities in turn increases the catalytic activity of PLC-γ isozymes which results in increased production of 1,4,5-IP_3 and *sn*-1,2-diacylglycerol (DAG).

D. Other Regulatory Proteins

Yet other regulatory proteins may be coexpressed with a heterologous phospholipase in yeast cells. Recent work has also focused on the interaction between cytoskeletal elements and polyphosphoinositides. For example PtdIns(4,5)P_2 has been found to bind to profilin. (Vojtek et al. (1991) *Cell* 66:497). PIP_2-binding proteins are known to bind actin and to regulate its assembly in vivo, suggesting a possible role for PIP_2 hydrolysis by PLC enzymes in the regulation of cytoskeletal organization, volume regulation, and in other cellular

functions related to the state of cytoskeletal assembly. Furthermore, Goldschmidt-Clermont et al. (1991) Science 251:1231 demonstrated a role for profilin in regulating PI turnover *in vivo* by showning that profilin-bound PIP_2 was found to be resistant to cleavage by PLC-γl except when the PLC-γl had been activated by tyrosine phosphorylation. Other exemplary molecules which may regulate a phospholipase include profilin, gelsonin, cofilin, alpha-actin, and villin. The binding sites for PtdIns(4,5)P 2 on gelsolin and villin have recently been identified (Janmey et al.,(1992) *J. Biol Chem* 267:11818).

Recent studies of PLC-δ regulation suggest that it may be regulated by a novel class of regulatory proteins (p122-RhoGAP) that show similarity to the GTPase activating protein homology region of bcr, display GAP activity towards RhoA, and bind to and directly stimulate PLC-δ1 (Homma, Y., and Y. Emori, 1995) Other reports suggest that rho can regulate PtdIns(4,5)P_2 levels (Chong et al. (1994) Cell 79:507).

Also some PLCs such as PLCβ1 (Bernstein and Ross) have a GAP-like activity for certain Gq family members. This activity serves to down regulate the activity of both the Gq and the PLC itself.

Other exemplary regulatory proteins include: IP3 receptors, calcium channels, DAG protein kinase C (PKC), PKA3 (cAMP dependent protein kinase catalytic subunit), SOK1 (suppressor of PKA overexpression), CMD1 (calmodulin, CMK1 (Calcium/calmodulin dependent protein kinase), and CMK2 (calmodulin dependent protein kinase).

V. Drug Screening

In certain embodiments, the assay is characterized by the use of recombinant cells to sample a variegated compound library for phospholipase agonists and/or antagonists. In preferred embodiments, the reagent cells express a mammalian phospholipase capable of producing a detectable signal in the reagent cell.

In certain embodiments, the reagent cell also produces the test compound which is being screened. For instance, the reagent cell can produce a test polypeptide, a test nucleic acid and/or a test carbohydrate which is screened for its ability to modulate the mammalian phospholipase activity. In such embodiments, a culture of such reagent cells will collectively provide a variegated library of potential phospholipase effectors and those members of the library which either agonize or antagonize the phospholipase function can be selected and identified. Moreover, it will be apparent that the reagent cell can be used to detect agents which directly alter the activity of the mammalian phospholipase, or which act on some target upstream or downstream of the mammalian phospholipase.

In other embodiments, the test compound is exogenously added. In such embodiments the test compound is contacted with the reagent cell. Exemplary compounds which can be screened for activity include peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries. In such embodiments, both compounds which agonize or antagonize the phospholipase function can be selected and identified. Moreover, it will be apparent that the reagent cell can be used to detect agents which directly alter the activity of the mammalian phospholipase, or which act on some target upstream or downstream of the mammalian phospholipase.

In still other embodiments, the test compound is produced by cells which are cocultured with the reagent cells expressing a mammalian phospholipase.

A. Screening and Selection: Assays of Second Messenger Generation

When screening for bioactivity of peptides, intracellular second messenger generation can be measured directly. Exemplary assays for the direct measurement of phospholipase generated second messengers are provided below; other assays for the measurement of second messengers generated in a signal transduction pathway involving a phospholipase will be apparent to those of skill in the art and may be substituted for the assays described below.

In one embodiment, the phospholipase enzymatic activity can be measured in yeast cell extracts. In other embodiments measurements of a detecting a phospholipase activity are made using intact yeast cells.

Certain receptors stimulate the activity of phospholipase C which stimulates the breakdown of phosphatidylinositol 4,5, bisphosphate to 1,4,5-IP_3 (which mobilizes intracellular Ca++) and diacylglycerol (DAG) (which activates protein kinase C). Inositol lipids can be extracted and analyzed using standard lipid extraction techniques. DAG can also be measured using thin-layer chromatography. Water soluble derivatives of all three inositol lipids (IP_1, IP_2, IP_3) can also be quantitated using radiolabelling techniques or HPLC.

The mobilization of intracellular calcium or the influx of calcium from outside the cell can be measured using standard techniques. The choice of the appropriate calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca^{++}-sensitive microelectrodes depends on the cell type and the magnitude and time constant of the event under study (Borle (1990) *Environ Health Perspect* 84:45-56). As an exemplary method of Ca^{++} detection, cells could be loaded with the Ca^{++}sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in Ca^{++} measured using a fluorometer.

The other product of PIP_2 breakdown, DAG can also be produced from phosphatidyl choline. The breakdown of this phospholipid in response to receptor-mediated signaling can also be measured using a variety of radiolabelling techniques.

The activation of phospholipase A2 can easily be quantitated using known techniques, including, for example, the generation of arachadonate in the cell.

In certain embodiments of the assay, it may be desirable to screen for changes in cellular phosphorylation. The ability of compounds to modulate phospholipase activation could be screened using colony immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430) using anti-phosphotyrosine. Reagents for performing such assays are commercially available, for example, phosphotyrosine specific antibodies which measure increases in tyrosine phosphorylation and phospho-specific antibodies can be purchased (New England Biolabs, Beverly, MA). Tests for phosphorylation could be useful to measure the phosphorylation of proteins in response to the modulation of a phospholipase activity; it is noted that protein phosphorylation need not be directly affected by the modulation in phospholipase activity, but rather may amplify signals generated by the phospholipase. Multi-kinase cascades allow not only signal amplification but also signal divergence to multiple effectors that are often cell-type specific, allowing a growth factor to stimulate mitosis of one cell and differentiation of another.

One such cascade is the MAP kinase pathway that appears to mediate both mitogenic, differentiation and stress responses in different cell types. Stimulation of growth factor receptors results in Ras activation followed by the sequential activation of c-Raf, MEK, and p44 and p42 MAP kinases (ERK1 and ERK2). Activated MAP kinase then phosphorylates many key regulatory proteins, including p90RSK and Elk-1 that are phosphorylated when MAP kinase translocates to the nucleus. Homologous pathways exist in mammalian and yeast cells. For instance, an essential part of the S. cerevisiae pheromone signaling pathway is comprised of a protein kinase cascade composed of the products of the STE11, STE7, and FUS3/KSS1 genes (the latter pair are distinct and functionally redundant). Accordingly, phosphorylation and/or activation of members of this kinase cascade can be detected and used to assay phospholipase modulation.

B. Complementation

Techniques to assay for complementation of a mutant endogenous yeast gene are well known in the art; numerous assays exist for the selection of auxotrophs. For example, as described in detail herein, conditional PLC1 mutations lead to a pleitropy of phenotypes expressed in yeast under nonpermissive conditions and have been complemented by

functional expression of mammalian PLCs. In one embodiment of the present assay it will be possible to test for agonists and/or antagonists of a functionally integrated, exogenous phospholipase by observing reversion to the original mutant phenotype. For example, yeast may cease to display a temperature or NaCl sensitive phenotype upon expression of a mammalian PLC, and may revert to a temperature or NaCl sensitive phenotype upon treatment with an antagonist of the exogenous PLC.

C. Screening and Selection Using Reporter Gene Constructs

Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on phospholipase signaling. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to phospholipase signal modulation, with the level of expression of the reporter gene providing the receptor-dependent detection signal. If only one transcriptional regulatory element is included it must be a regulatable promoter. At least one the selected transcriptional regulatory elements must be indirectly or directly regulated by the activity of the heterologous phospholipase whereby activity of the phospholipase can be monitored via transcription of the reporter genes.

Many reporter genes and transcriptional regulatory elements are known to those of skill in the art and others may be identified or synthesized by methods known to those of skill in the art.

A reporter gene includes any gene that expresses a detectable gene product, which may be RNA or protein. Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties.

Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368).

Transcriptional control elements which may be included in a reporter gene construct include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is rapidly induced, generally within minutes, after modulation of a phospholipase. Examples of such genes include those which are responsive to the second messengers generated upon phospholipase activation, such as Ca^{++}, cAMP, and others. One such gene is c-fos, an immediate early gene (see, Sheng et al. (1990) Neuron 4: 477-485). Immediate early genes are genes that are rapidly induced upon binding of a ligand to a cell surface protein. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular simulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, specific mRNA expression may be detected using Northern blots or specific protein product may be identified by a characteristic stain or an intrinsic activity.

In preferred embodiments, the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence.

The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound or it may be compared with the amount of transcription in a substantially identical cell that lacks heterologuos DNA, such as the phospholipase. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the activity of the phospholipase.

In other preferred embodiments, the reporter or marker gene provides a selection method such that cells in which the peptide is a ligand for the receptor have a growth advantage. For example the reporter could enhance cell viability, e.g., by relieving a cell nutritional requirement, and/or provide resistance to a drug. For example the reporter gene could encode a gene product which confers the ability to grow in the presence of a selective agent, e.g., canavanine.

For example,use of the *S. cerevisiae plc1*Δ allele as an endogenous mutant phospholipase C gene provides a readout system, whereby functional complementation (i.e. detection of the second detectable phenotype rather than the first detectable phenotype) can be detected or quantitated by cell growth. However, growth systems suffer from their "quantal" nature. Other readout systems, which provide a different detectable phenotype when the endogenous, mutant phospholipase C is complemented by the expression of heterologous phospholipase C may be more sensitive in a given range or may be more convenient for automation. For example, a transcriptionally based readout may be preferred because it is rapid (i.e. hours instead of days) and affords greater flexiblity both in terms of types of readout and dynamic range of the readout. For example, by placing the bacterial gene encoding lacZ under the control of the promoter sensitive to signals generated upon activation of a phospholipase pathway can be detected in less than an hour by monitoring the ability of permeabilized yeast to produce color from a chromogenic substrate. The rapidity of such a readout would, in itself, be advantageous. And such a readout would be necessary to monitor phospholipase activity under conditions where the yeast do not grow.

A transcriptional readout can be developed by identifying genes/promoters that are positively or negatively regulated by the PLC1 locus (i.e. promoters that are up or down-regulated in PLC1 vs. *plc1*Δ strains or in *plc1*Δ strains expressing human PLC-β2 plus Gα 16). These promoters can then be linked to a reporter gene. Alternatively, the reporter gene can be expressed in response to a downstream phospholipase C signal molecule, such as IP3, DAG, increased intracellular calcium levels or increased PKC activity.

For example, a transcriptional based readout can be constructed using yeast expressing mammalian PLC enzymes in the following manner. CREB is a transcription factor whose activity is regulated by phosphorylation at a particular serine (S133). When this serine residue is phosphorylated, "phospho-CREB", binds to a recognition sequence known as a CRE (cAMP Responsive Element) found to the 5' of promotors known to be responsive to elevated cAMP levels. Upon binding of phosphorylated CREB to a CRE, transcription from this promoter is increased. Phosphorylation of CREB is seen in response to both increased cAMP levels and increased intracellular calcium levels. Increased cAMP levels result in activation of protein kinase A, which in turn phosphorylates CREB and leads to binding to CRE and transcriptional activation. Increased intracellular calcium levels results in activation of calcium/calmodulin responsive kinases (CaM kinases). Phosphorylation of CREB by CaM kinase IV is effectively the same as phosphorylation of CREB by PKA, and results in transcriptional activation of CRE containing promotors.

Therefore, a transcriptional-based readout can be constructed in plc1Δ yeast strains expressing a mammalian PLC enzyme, regulatory proteins (G α and/or Gβγ), if necessary,

and/or a reporter gene (i.e. lacZ) whose expression is driven by a basal promoter containing one or more CREs in the following manner. Changes in the intracellular concentration of Ca^{++} (a result of alterations in the activity of the recombinant PLC) will result in changes in the level of expression of the reporter gene if: a) CREB is also co-expressed in the cell, and b) either the endogenous yeast CaM kinase will phosphorylate CREB in response to increases in calcium or if an exogenously expressed CaM kinase IV is present in the same cell. In other words, stimulation of PLC activity will result in phosphorylation of CREB and increased transcription from the CRE-lacZ construct, while inhibition of PLC activity will result in decreased transcription from the CRE-lacZ construct.

In one embodiment, the promoter is activated upon activation of the phospholipase, in which case, for selection, the expression of the marker gene should result in a benefit to the cell. A preferred marker gene is the imidazoleglycerol phosphate dehydratase gene (HIS3). If a phospholipase responsive promoter is operably linked to a beneficial gene, the cells will be useful in screening or selecting for phospholipase activators. If it is linked to a deleterious gene, the cells will be useful in screening or selecting for inhibitors.

Alternatively, the promoter may be one which is repressed by phospholipase, thereby preventing expression of a product that is deleterious to the cell. With a phospholipase-repressed promoter, one screens for agonists by linking the promoter to a deleterious gene, and for antagonists, by linking it to a beneficial gene.

Repression may be achieved by operably linking a phospholipase-induced promoter to a gene encoding mRNA that is antisense to at least a portion of the mRNA encoded by the marker gene (whether in the coding or flanking regions), so as to inhibit translation of that mRNA. Repression may also be obtained by linking a phospholipase induced promoter to a gene encoding a DNA-binding repressor protein, and incorporating a suitable operator site into the promoter or other suitable region of the marker gene.

In yeast, suitable positively selectable (beneficial) genes include the following: *URA3, LYS2, HIS3, LEU2, TRP1; ADE1,2,3,4,5,7,8; ARGI, 3, 4, 5, 6, 8; HIS1, 4, 5; ILV1, 2, 5; THR1, 4; TRP2, 3, 4, 5; LEU1, 4; MET2,3,4,8,9,14,16,19; URA1,2,4,5,10; HOM3,6; ASP3; CHO1; ARO 2,7; CYS3; OLE1; IN01,2,4; PR01,3* Countless other genes are potential selective markers. The above are involved in well-characterized biosynthetic pathways. The imidazoleglycerol phosphate dehydratase (IGP dehydratase) gene (HIS3) is preferred because it is both quite sensitive and can be selected over a broad range of expression levels. In the simplest case, the cell is auxotrophic for histidine (requires histidine for growth) in the absence of activation. Activation leads to synthesis of the enzyme and the cell becomes prototrophic for histidine (does not require histidine). Thus the selection is for growth in the

absence of histidine. Since only a few molecules per cell of IGP dehydratase are required for histidine prototrophy, the assay is very sensitive.

In appropriate assays, so-called counterselectable or negatively selectable genes may be used. Suitable genes include: URA3 (orotidine-5'-phosphate decarboxylase; inhibits growth on 5-fluoroorotic acid), LYS2 (2-aminoadipate reductase; inhibits growth on α-aminoadipate as sole nitrogen source), CYH2 (encodes ribosomal protein L29; cycloheximide-sensitive allele is dominant to resistant allele), CAN1 (encodes arginine permease; null allele confers resistance to the arginine analog canavanin), and other recessive drug-resistant markers.

The marker gene may also be a screenable gene. The screened characteristic may be a change in cell morphology, metabolism or other screenable features. Suitable markers include beta-galactosidase (Xgal, C_{12}FDG, Salmon-gal, Magenta-Gal (latter two from Biosynth Ag)), alkaline phosphatase, horseradish peroxidase, exo-glucanase (product of yeast exbl gene; nonessential, secreted); luciferase; bacterial green fluorescent protein; (human placental) secreted alkaline phosphatase (SEAP); and chloramphenicol transferase (CAT). Some of the above can be engineered so that they are secreted (although not β-galactosidase). A preferred screenable marker gene is beta-galactosidase; yeast cells expressing the enzyme convert the colorless substrate Xgal into a blue pigment. Again, the promoter may be receptor-induced or receptor-inhibited.

D. Detection of Inhibition or Activation of Proteins Other Than Phospholipase

The present invention also facilitates the detection of inhibitors or activators of proteins other than phospholipase provided that the yeast cell expresses or is engineered to express the protein of interest in such a manner that it is functionally "coupled", directly or indirectly, to the phospholipase.

For example, the yeast cells can also be used in more general readout systems for modulation of the activity of a variety of different cellular signalling components which regulate phospholipase activation, such as receptors, which are known to couple to phospholipases, either directly, (e.g. the PDGF receptor) or via a regulatory protein (e.g. a G-protein coupled receptor or tyrosine kinase coupled receptor); a G-protein heterotrimer; a G α subunit; a Gβγ subunit; another protein known to interact functionally with a known phospholipase substrate (e.g., profilin, gelsolin, cofilin, and alpha-actin); and other effectors whose activity is dependent on phospholipase C activity (i.e., IP3 receptors/calcium channels,

other potential IP3 sensitive calcium channels, and other calcium sensitive enzyme activities such as PKC or CaM kinase).

In a preferred embodiment the yeast cells of the present invention may be used to identify drugs which modulate the activity of a mammalian G protein-coupled receptor. In this embodiment, the yeast cell is engineered to express a mammalian G protein-coupled receptor.

E. Test Compounds

Exemplary compounds which can be screened for activity include peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries.

One class of potential modulators of particular interest is the peptide class. The term "peptide" is used herein to refer to a chain of two or more amino acids, with adjacent amino acids joined by peptide (-NHCO-) bonds. Thus, the peptides of the present invention include oligopeptides, polypeptides, and proteins. Preferably, the peptides of the present invention are 2 to 200, more preferably 5 to 50, amino acids in length. The minimum peptide length is chiefly dictated by the need to obtain sufficient potency as an activator or inhibitor. The maximum peptide length is only a function of synthetic convenience once an active peptide is identified.

Synthetic peptides are also of interest. By way of example, peptides based on the calmodulin-binding domain of calmodulin-dependent phospholipases could serve as modulators of phospholipase activity. In addition, peptides or molecules of any structure which inhibit the interaction between the phospholipase and known endogenous modulators of phospholipase activity are of interest. Known endogenous phospholipase modulators include Ca2+, Ca2+/calmodulin, protein kinase C, protein kinase A, Gαs, Gαi, Gβγ, and adenosine.

When peptide drugs are being assayed, the yeast cells may be engineered to express the peptides, rather than being exposed to the peptides simply by adding the peptides to the culture medium. Peptide libraries are systems which simultaneously display, in a form which permits interaction with a target, a highly diverse and numerous collection of peptides. These peptides may be presented in solution (Houghten 1991), or on beads (Lam 1991), chips (Fodor 1991), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull 1992) or on phage (Scott, Devlin, Cwirla, Felici, Ladner '409). Many of these systems are limited in terms of the maximum length of the peptide or the composition of the peptide (e.g., Cys excluded). Steric factors, such as the proximity of a support, may interfere with binding. Usually, the screening is for binding in vitro to an artificially presented target, not for

activation or inhibition of a cellular signal transduction pathway in a living cell. While a cell surface receptor may be used as a target, the screening will not reveal whether the binding of the peptide caused an allosteric change in the conformation of the receptor.

Ladner, USP 5,096,815 describes a method of identifying novel proteins or polypeptides with a desired DNA binding activity. Semi-random ("variegated") DNA encoding a large number of different potential binding proteins is introduced, in expressible form, into suitable host cells. The target DNA sequence is incorporated into a genetically engineered operon such that the binding of the protein or polypeptide will prevent expression of a gene product that is deleterious to the cell under selective conditions. Cells which survive the selective conditions are thus cells which express a protein which binds the target DNA. While it is taught that yeast cells may be used for testing, bacterial cells are preferred. The interactions between the protein and the target DNA occur only in the cell, not in the periplasm, and the target is a nucleic acid, not a protein.

Substitution of random peptide sequences for functional domains in cellular proteins permits some determination of the specific sequence requirements for the accomplishment of function. Though the details of the recognition phenomena which operate in the localization of proteins within cells remain largely unknown, the constraints on sequence variation of mitochondrial targeting sequences and protein secretion signal sequences have been elucidated using random peptides (Lemire et al., J. Biol. Chem. 264, 20206 (1989) and Kaiser et al. Science 235, 312 (1987), respectively).

Yeast have been engineered to express foreign polypeptide variants to be tested as potential antagonists of mammalian receptors. Libraries encoding mutant glucagon molecules were generated through random misincorporation of nucleotides during synthesis of oligonucleotides containing the coding sequence of mammalian glucagon. These libraries were expressed in yeast and culture broths from transformed cells were used in testing for antagonist activity on glucagon receptors present in rat hepatocyte membranes (Smith et al. 1993).

In one embodiment, the yeast cells are engineered to express a peptide library. A "peptide library" is a collection of peptides of many different sequences (typically more than 1000 different sequences), which are prepared essentially simgrowthultaneously, in such a way that, if tested simultaneously for some activity, it is possible to characterize the "positive" peptides. The peptide library of the present invention takes the form of a yeast cell culture, in which essentially each cell expresses one, and usually only one, peptide of the library. While the diversity of the library is maximized if each cell produces a peptide of a different sequence, it is usually prudent to construct the library so there is some redundancy. Moreover, each sequence should be produced at assayable levels.

The peptides of the library are encoded by a mixture of DNA molecules of different sequence. Each peptide-encoding DNA molecule is ligated with a vector DNA molecule and the resulting recombinant DNA molecule is introduced into a yeast cell. Since it is a matter of chance which peptide-encoding DNA molecule is introduced into a particular cell, it is not predictable which peptide that cell will produce. However, based on a knowledge of the manner in which the mixture was prepared, one may make certain statistical predictions about the mixture of peptides in the peptide library.

It is convenient to speak of the peptides of the library as being composed of constant and variable residues. If the nth residue is the same for all peptides of the library, it is said to be constant. If the nth residues varies, depending on the peptide in question, the residue is a variable one. The peptides of the library will have at least one, and usually more than one, variable residue. A variable residue may vary among any of two to any of all twenty of the genetically encoded amino acids; the range of possibilities may be different, if desired, for each of the variable residues of the peptide. Moreover, the frequency of occurrence of the allowed amino acids at particular residue positions may be the same or different. The peptide may also have one or more constant residues.

There are two principal ways in which to prepare the required DNA mixture. In one method, the DNAs are synthesized a base at a time. When variation is desired, at a base position dictated by the Genetic Code, a suitable mixture of nucleotides is reacted with the nascent DNA, rather than the pure nucleotide reagent of conventional polynucleotide synthesis.

The second method provides more exact control over the amino acid variation. First, trinucleotide reagents are prepared, each trinucleotide being a codon of one (and only one) of the amino acids to be featured in the peptide library. When a particular variable residue is to be synthesized, a mixture is made of the appropriate trinucleotides and reacted with the nascent DNA.

Once the necessary "degenerate" DNA is complete, it must be joined with the DNA sequences necessary to assure the expression of the peptide, as discussed in more detail elsewhere, and the complete DNA construct must be introduced into the yeast cell.

VI. Periplasmic Secretion

The cytoplasm of the yeast cell is bounded by a lipid bilayer called the plasma membrane. Between this plasma membrane and the cell wall is the periplasmic space. Peptides secreted by yeast cells cross the plasma membrane through a variety of mechanisms and thereby enter the periplasmic space. The secreted peptides are then free to interact with

other molecules that are present in the periplasm or displayed on the outer surface of the plasma membrane. The peptides then either undergo re-uptake into the cell, diffuse through the cell wall into the medium, or become degraded within the periplasmic space.

The peptide library may be secreted into the periplasm by one of two distinct mechanisms, depending on the nature of the expression system to which they are linked. In one system, the peptide may be structurally linked to a yeast signal sequence, such as that present in the α-factor precursor, which directs secretion through the endoplasmic reticulum and Golgi apparatus. Since this is the same route that the receptor protein follows in its journey to the plasma membrane, opportunity exists in cells expressing both the receptor and the peptide library for a specific peptide to interact with the receptor during transit through the secretory pathway. This has been postulated to occur in mammalian cells exhibiting autocrine activation. Such interaction would likely yield activation of the linked pheromone response pathway during transit, which would still allow identification of those cells expressing a peptide agonist.

An alternative mechanism for delivering peptides to the periplasmic space is to use the ATP-dependent transporters of the STE6/MDR1 class. This transport pathway and the signals that direct a protein or peptide to this pathway are not as well characterized as is the endoplasmic reticulum-based secretory pathway. Nonetheless, these transporters apparently can efficiently export certain peptides directly across the plasma membrane, without the peptides having to transit the ER/Golgi pathway. We anticipate that at least a subset of peptides can be secreted through this pathway by expressing the library in context of the a-factor prosequence and terminal tetrapeptide. The possible advantage of this system is that the receptor and peptide do not come into contact until both are delivered to the external surface of the cell. Thus, this system strictly mimics the situation of an agonist or antagonist that is normally delivered from outside the cell. Use of either of the described pathways is within the scope of the invention.

The present invention does not require periplasmic secretion, or, if such secretion is provided, any particular secretion signal or transport pathway.

VII. Pharmaceutical Preparations of Identified Agents

After identifying certain test compounds as potential modulators of the target phospholipase activity, the practioner of the subject assay will continue to test the efficacy and specificity of the selected compounds both *in vitro* and *in vivo*. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, agents identified in the

subject assay can be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human.

The subject compounds selected in the subject, or a pharmaceutically acceptable salt thereof, may accordingly be formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the compound, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations". Based on the above, such pharmaceutical formulations include, although not exclusively, solutions or freeze-dried powders of the compound in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered media at a suitable pH and isosmotic with physiological fluids. In preferred embodiment, the compound can be disposed in a sterile preparation for topical and/or systemic administration. In the case of freeze-dried preparations, supporting excipients such as, but not exclusively, mannitol or glycine may be used and appropriate buffered solutions of the desired volume will be provided so as to obtain adequate isotonic buffered solutions of the desired pH. Similar solutions may also be used for the pharmaceutical compositions of compounds in isotonic solutions of the desired volume and include, but not exclusively, the use of buffered saline solutions with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH).

The present invention is further illustrated by the following examples, which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Exemplification

Example 1. Development of Yeast Strains and Complementation Assays

Methods

A. Yeast Plasmids Which Express G Proteins

Expression Plasmids

All expression plasmids are based upon the shuttle vectors originally described by Sikorski and Hieter (R.S. Sikorski and P.Hieter (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122:19-27). The expression plasmids with the GPA1 promoter (Cadus 1127), the PGK promoter (Cadus 1447) and the CUP1 promoter (Cadus 1725 and 1728) have been described elsewhere (Cadus published Patent Application WO 94/23025 entitled "Yeast cells engineered to produce pheromone system protein surrogates, and uses therefor).

Gα-subunits - Construction of Low Copy Expression Plasmids

The following full length G-protein encoding inserts: GαS, Gαi2, Gαi3, Gαq, Gα11, Gα16, GαOA, GαOB, Gα12, GNAZ, and activated alleles (GTPase-deficient) of GαS, Gα i2, Gαq, and Gα16 (Wu et al. (1992); Wu et al (1993); Park et al (1992) and references therein) were amplified by polymerase chain reaction using VENT polymerase, with cloning sites added to the 5' and 3' ends of the coding regions. In all cases, the restriction site added to the 5' end of the gene encoded a novel NcoI compatible overhang, while the restriction site added to the 3' end of the gene encoded a novel XhoI compatible overhang. PCR products were gel purified, restricted with the appropriate endonucleases, and cloned into NcoI/XhoI cut Cadus 1127 (construction of this plasmid is described in Cadus patent application WO94/23025). Cadus 1127 is a low copy plasmid (CEN6 ARS4) in which heterologous gene expression is driven by the GPA1 promoter (-1400 to +1 of the native GPA1 locus). A novel NcoI site has been engineered into this vector such that the initiator methionine (at position +1) is embedded in this site (CCATGG).

All G-protein expression cassettes were constructed as described below for Gα16. Full length human Gα16 was amplified from Cadus plasmid 1127 with Vent polymerase (New England Biolabs) using 5' oligonucleotide KS9829 = 5' GGGCGTCTCACATGGCCCGCTCGCTGACC 3' (in which a BsmB1 site encoding a NcoI overhang (CATG) has been introduced and is underlined) and 3' oligonucleotide KS9830 = 5' GGGCTCGAGCTGGGTCACAGCAGGTGGATC 3' (in which an XhoI site has been introduced following the termination codon (underlined). Template (100 ng) and primers (1 uM final concentrations) were added in the presence of 10x reaction buffer containing 2 mM MgSO4. Template was denatured at 94°C for 30 seconds in the presence of primers followed byaddition of 1ul per 100ul reaction volume of VENT polymerase. DNA was amplified in a two stage amplification protocol: Stage 1 = 2 cycles of 94°C/ 30 seconds followed by 55°C/30 seconds followed by 72°C/60 seconds; Stage 2 = 25 cycles of 94°C/ 30 seconds followed by 60°C/30 seconds followed by 72°C/ 60 seconds. The amplified product was purified by gel electrophoresis, cut with BsmBI and XhoI, and ligated to NcoI / XhoI cut and phosphatase treated Cadus 1127 vector DNA. Recombinant clones were identified by colony PCR using primers flanking the inserted Gα16 sequences, confirmed by restriction digestion, and validated by dideoxynucleotide sequencing of the PCR product. Gαq and Gα11 expressing plasmids were prepared in a similar fashion using the following oligonucleotide primer pairs for amplification: Gαq - 5' oligonucleotide = KS A14282 = 5' GGGCGTCTCACATGACTCTGGAGTCCATCATG 3' (encoding an Esp3I site at the 5' end which leaves an NcoI overhang following cleavage with Esp3I or BsmBI) and 3' oligonucleotide = KS A 14283 = 5'GGGCGTCTCCTCGAGGCACGGTTAGACCAG 3' (encoding an Esp3I site at the 3' end that leaves an XhoI compatible overhang following cleavage with Esp3I or BsmI). Gα11 - 5' oligonucleotide = KS A14645 = 5'GGGGGTCTCCCATGACTCTGGAGT- CCATGATG3' (encoding a BsaI sites that creates an NcoI compatible overhang following cleavage with BsaI) and 3' oligonucleotide = KS A14285 = 5' GGGCTCGAGG-AAGCCTGGCCCT 3' (encoding a XhoI site).

Gα-subunits - High Copy Expression Plasmids

Full length G-protein encoding inserts were amplified by polymerase chain reaction using VENT polymerase as described above, with cloning sites added to the 5' and 3' ends of the coding regions. In all cases, the restriction site added to the 5' end of the gene encoded a novel NcoI compatible overhang, while the restriction site added to the 3' end of the gene encoded a novel XhoI compatible overhang. PCR products were gel purified, restricted with the appropriate endonucleases, and cloned into NcoI/XhoI cut Cadus 1447. Cadus 1447 is a high copy plasmid (2 micron origin of replication) in which heterologous gene expression is

driven by the phosphoglycerol kinase (PGK) promoter. A novel NcoI site is present at the initiator methionine, and a unique XhoI site is downstream from it. G-proteins cloned in this manner include GαS, Gαq, Gα11, Gα16, GαOA, GαOB, Gα12, GNAZ, and activated alleles (GTPase-deficient) of GαS, Gαi2, Gαq, and Gα16.

Yeast Plasmids Which Express Phospholipase C Genes

PLC-β1

Rat PLC-β1 (Suh et al., (1988)) was cloned into Cadus 1725 (CUPp LEU2 CEN ARS AmpR) and Cadus 1728 (CUPp LEU2 2 micron origin AmpR). In these expression vectors, a minimal CUP1 promoter has been engineered to contain an initiator methionine codon (ATG) imbedded in an NcoI site, so that cloning into the NcoI cut vector introduces the gene of interest in frame into the NcoI site. The resulting plasmid, Cadus 1642 (Rat PLC-β1), was cleaved with ApaLI and ligated to a duplex adapter encoding the amino terminal seven residues of rat PLC-β1 with yeast codon bias. The sequences of these oligonucleotides are: Oligo A = 5' CATGGCTGGTGCTCAACCAGGTG 3' and Oligo B = 5' TGCACACCTGG-TTGAGCACCAGC 3'. Oligo B was kinased with polynucleotide kinase prior to annealing and ligation. Following ligation to ApaLI cut Cadus plasmid 1642, the ligation mixture was treated with polynucleotide kinase, cleaved with BamHI and a 3.8 kb fragment with the entire coding region of rat PLC-β1 was isolated by gel electrophoresis. This 3.8 kb PLC-β1 fragment was then ligated to NcoI - BamHI cut Cadus 1725 and Cadus 1728. Plasmids containing inserts in the correct orientation were identified by colony PCR and verified by restriction analysis and double-stranded DNA sequencing using Sequenase V2.0 (U.S. Biochemicals).

PLC-β2

Human PLC-β2 (Park et al., (1992) was cloned into Cadus plasmid 1725 (CUPp LEU2 CEN ARS AmpR) and Cadus 1728 (CUPp LEU2 2 micron origin AmpR). The resulting plasmid, Cadus 1680 (Human PLC-β2), was restricted with Eco47III and EcoRI to completion and then treated with T4 polymerase to fill in the EcoRI overhang. The EcoRI-blunt/Eco47III cut plasmid was then ligated to a duplex adapter encoding the amino terminal twenty-one residues of human PLC-β2 with yeast codon bias. The sequences of these are:

Oligo C = 5'CATGTCTTTGTTGAACCCAGTTTTGTTGCCACCAAAGGTTAAGGCTT-ACTTGTCTCAAGGTGAGC 3'

and Oligo D = 5'GCTCACCTTGAGACAAGTAAGCCTTAACCTTTGGTGGCAACAAA-ACTGGGTTCAACAAAGA3'.

Oligo D was treated with polynucleotide kinase prior to annealing to oligo C and ligation to EcoRI-blunt/Eco47III cut Cadus 1680. Following ligation, the ligation mixture was treated with polynucleotide kinase to phosphorylate the NcoI overhang at the 5' end of the gene and the 3.8 kb fragment with the entire coding region of human PLC-β2 was purified by gel electrophoresis. This 3.8 kb fragment has NcoI compatible overhangs at both termini, and was ligated to NcoI cut/ shrimp alkaline phosphatase treated Cadus 1725 and 1728. Plasmids containing insert in the correct orientation were identified by colony PCR and verified by restriction analysis and double-stranded DNA sequencing using Sequenase V2.0.

Table 3

Yeast Strains

No.	MAT	Genotype	NAME	P1GENE	P2 Name	P2GENE
1630	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52				
1632	α	PLC1 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52`				
1633	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52				
1901	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1443	URA3 2mu-ori REP3 AmpR PGKp		
1902	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1460	URA3 CEN6 ARS4 AmpR PGKp		
1903	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1637	GAL1p PLC1 URA3 2mu-ori AmpR		
1904	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR		
2099	α	plc1*2 :LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-51	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1127	TRP1 CEN6 ARS4 AmpR GPA1p
2100	α	plc1*2..LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	J639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1179	TRP1 CEN6 ARS4 AmpR GPA1p GPA1
2101	α	plc1*2::LEu2 ade2-101 his3*200 leu2*a lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1181	TRP1 CEN6 ARS4 AmpR GPA1pGaS
2102	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 frp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1606	trp1 CEN6 ARS4 AmpR GPA1Pgα2

2103	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1617	TRP1 CEN6 ARS4 AmpR GPA1p Human Gα16
2104	α	PLC1*2::leu2 ADE2-101 HIS3*200 LEU2*1 LYS1-801 TRP1*1 URA3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1685	TRP1 CEN6 ARS4 AmpR GPA1p Murine Gαq
2105	α	plc1*2::LEU2 DE2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1749	TRP1 CEN6 ARS4 AmpR GPA1p Murine Gαq
2106	α	plc1*2::LEU2 ade2-101 his3*200leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpRq\	1753	TRP1 CEN6 ARS4 AmpR GPA1p Human Gα16-Q212L
2107	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1447	TRP1 1mu-ori AmpR PGKp
2108	α	plc1*2::LEu2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta URA3 CEN6 ARS4 AmpR	1630	TRP1 2mu-ori AmpR PGKp GαS
2110	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-BETA1 ura3 cen6 ars4 aMPr	1783	TRP1 2mu-ori AmpR PGKp Murine Gαq
2111	α	plc1*2::LEu2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1786	TRP1 2mu-ori AmpR PGKp Human Gα 16
2112	α	plc1*2::LEU2 ade2-101 his3*200 leu2*a lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1786	TRP1 2mu-ori AmpR PGKp Human Gα q-Q209L
2113	α	plc1*2::LEU2 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta1 URA3 CEN6 ARS4 AmpR	1786	TRP1 2mu-ori AmpR PGKp Human Gα 116-Q212L
2945	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1725	LEU2 CEN6 ARSH4 CUP1 AmpR	1127	TRP1 GPA1p CEN6 ARS4 AmpR
2946	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1728	LEU2 2m-ori REP3 CUP1 AmpR	1855	GPA1p BamHI GPA-Gα16 TRP1 CEN6 ARS4 AmpR
2948	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1728	LEU2 2mu-ori REP3 CUP1 AmpR	1855	GPA1p BamHI GPA-Gα16 TRP1 CEN6 ARS4 AmpR
2950	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1639	GAL1p rat PLC-beta-1 URA3 CEN ARS AmpR	1127	TRP1 GPA1p CEN6 ARS4 AmpR
2952	α	plc1*1::HIS3 ade2-101 his3*200 leu2*a lys2-801 trp1*1 ura3-52	1640	ADH1p rat PLC-beta-1 LEU2 2mu origin AmpR	1855	GPA1p BamHI GPA-Gα16 TRP1 CEN6 ARS4 AmpR

2954	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1922	CUP rat PLC-beta1 LEU2 2mu-ori REP3 AmpR	1127	TRP1 PA1p CEN6 ARS4 AmpR
2956	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1922	CUP rat PLC-beta1 LEU2 2mu-ori REP3 AmpR	1855	GPA1p BamHI GPA-Gα16 TRP1 CEN6 ARS4 AmpR
2958	α	plc1*1::HIS3 ade-2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1127	TRP1 GPA1p CEN6 ARS4 AmpR
2962	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta	1537	TRP1 2mu-ori REP3 AmpR PGKp-ratGαsQ227L
2964	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1617	GPA1p Human α 16 TRP1 CEN6 ARS4 AmpR
2966	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1685	GPA1pMurineGαq TRP1 CEN6 ARS4 AmpR f1 ori
2968	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1752	GPA1p-Gαq-Q209L TRP1 CEN6 ARS4 AmpR
2970	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1783	GPA1p-Gα16-Q212L TRP1 CEN6 ARS4 AmpR
2972	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1783	Murine Gαq PGKp TRP1 2mu ori REP3 AmpR
2974	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1786	PGKp Human α16 TRP1 2mu ori REP3 AmpR
2977	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1796	PGKp Human α16-!212L 2mu ori REP3 AmpR
2979	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC0 beta2	1794	PGKp Murine Gα 1-Q209L TRP1 2mu ori REP3 AmpR
2981	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC0 beta2	1850	PGKp Murine Gα 11 TRP1 2mu-ori REP3 AmpR f1 ori

3222	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1524	TRP1 GPA1p-ratG αs-Q227L CEN6 ARS4 AmpR
3226	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1606	GPA1p Human α i2 TRP1 CEN6 ARS4 AmpR
3228	α	plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1612	GPA1p Human α i3 TRP1 CEN6 ARS4 AmpR
3230	α	plc1*1::HIS3 ae2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC0 beta2	1622	GPA1p Murine α Oa TRP1 CEN6 ARS4 AmpR
3232	α	plc1*1::HIS3 ade2-101 his3*200 leu2*a lys2-801 trp1*1 ura3-52	1961	LEU2 CEN6 ARSH4 AmpR CUP-PLC-beta2	1623	GPA1p Murine α Ob TRP1 CEN6 ARS4 AmpR

Biological Deposit

S.cerevisiae strain CY 2964 (plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52 [LEU2 CEN6 ARS4 AmpR CUP-PLC-beta2], [GPA1p Human alpha16 TRP1 CEN6 ARS4 AmpR] was deposited with the American Type Culture Collection (Rockville, Maryland) on June 5, 1995 and has been designated with the ATCC Accession Number: 74345; and

S.cerevisiae strain CY 2954 (plc1*1::HIS3 ade2-101 his3*200 leu2*1 lys2-801 trp1*1 ura3-52 [CUP RAT PLCbeta1 LEU2 2mu-ori REP3 AmpR], GPA1p TRP1 CEN6 ARS4 AmpR] was deposited with the American Type Culture Collection (Rockville, Maryland) on June 5, 1995 and has been designated with the ATCC Accession Number: 74343.

PLC1Δ Yeast Strains and Complementation Assays

Yeast Transformations

All yeast transformation with the *plc1Δ* strains (Flick and Thorner (1993) were done using lithium acetate-based protocols and the Yeastmaker transformation kit (Clontech) according to the manufacturer's specifications. In general, strains were grown up overnight

at 30°C to late log phase and diluted the next morning to an OD600 = 0.2-0.3. Following 5 hours of growth at 30°C, cells were washed once in water and resuspended in lithium acetate/TE such that the cells were concentrated 30-50 fold. To 100 ul of this suspension of cells, carrier DNA (100 ug), and plasmid DNA (0.1 - 0.5 ug) were added. Cells and DNA were then vortexed, PEG/lithium acetate/TE added, the suspension vortexed again, and the suspension incubated at 30°C for 30 minutes. DMSO was then addedto a final concentration of 10% was then added and the cells were heat shocked at 42°C for 15 minutes. Following cooling on ice, the cells were centrifuged, the PEG containing supernatant was decanted, the cells were resuspended in sterile TE and plated on selective plates.

Growth assays and alternative readouts

Standard media were used for the culture of yeast cells, and established procedures were used for genetic manipulations (Rose, M.D., Winston, F., and P. Heiter (1990) Methods in Yeast Genetics: A Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). All yeast strains were grown under selective conditions on synthetic medium supplemented with nutrients appropriate for selection and maintenance of insertional mutations and plasmids (Guthrie, C, and G.R. Fink (1991) Methods in Enzymology Volume194. Guide to Yeast Genetics and Molecular Biology, Academic Press, Inc.). Strains to be tested for suppression of *plc1*Δ conditional lethal phenotypes were picked from single colonies from selective Synthetic Complete (SC) plates and were then streaked onto SC plates to single colonies and then grown for 48 to 72 hours under the following conditions.

Permissive growth conditions:

SC / 2% Glucose / 30°C

Non-permissive growth conditions:

SC / 2% Glucose / 1.2 M Sorbitol / 30°C
SC / 2% Glucose / 0.5 M NaCl / 30°C
SC / 2% Glucose / 37°C
SC / 2% Glucose / 12M Sorbitol / 37°C
SC / 2% Glucose / 0.5 M NaCl / 37°C

The non-permissive conditions are listed above from least stringent to most stringent.
Cell growth was monitored visually by light microscopy using a Nikon phase contrast microscope and were photographed using the UVP digital image processing system.

Results

GAL1 promoter driven PLC-β1 does not complement the plc1 mutant

phenotype

Initial attempts to complement the *plc1* ts and osmotic sensitivity phenotypes with mammalian PLC-β isozymes were carried out using a rat PLC-β1 construct driven by the GAL1 promoter (the sequence of this construct was verified by dideoxynucleotide sequencing) in Cadus yeast strains CY1630 (plc1Δ1::HIS3 ade2-101(Oc) his3-d100 leu2-d1 lys2-801 (Am) trp1-d1) and CY1633 (plc1Δ2::HIS3 ade2-101(Oc) his3-d100 leu2-d1 lys2-801 (Am) trp1-d1). In these experiments, no complementation was observed under conditions in which the GAL1 promoter was either repressed (in the presence of glucose) or when it was derepressed in the presence of low glucose and activated in the presence of galactose. In control experiments using a GAL1 promoter driven PLC1 cassette (Cadus plasmid 1639) transformed into the same strain backgrounds (CY1630 and CY1633), both the temperature sensitive and osmotic sensitivity phenotypes were complemented. These negative results with the rat PLC-β1 indicated that functional complementation of *plc1* phenotypes requires more than expression from the GAL1 promoter of the rat PLC-β1 gene alone.

Complementation Analysis with Gα subunits

Phospholipase-C β dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5, trisphosphate (IP_3) and diacylglycerol (DAG) is stimulated by both Gα and Gβγ subunits of heterotrimeric G-proteins. Stimulation of PLC-β activity by G α subunits is restricted to members of the Gq family that includes Gq, -11, -14, -15, and -16. Gα subunits belonging to other families (-s, -i, and -12/13) do not stimulate PLC-β dependent hydrolysis of PIP_2 . In several cell types, agonist dependent stimulation of IP_3 and calcium mobilization have been shown to be partially sensitive to pertussis toxin. This observation initially lead to speculation that Gi or Go could stimulate PLC-β activity. More recently the pertussis toxin sensitive component of agonist dependent IP_3 production has been shown to be due to the ability of Gβγ subunits to stimulate PLC-β isoforms directly.

In an effort to recapitulate G-protein dependent regulation of PLC-β isoforms in yeast, a series of CY1630 derivatives were transformed with expression vectors coding for mammalian G-proteins and complementation of the *plc1* mutant phenotypes were scored. Cadus yeast strains CY1901, CY1903 and CY1904 (all CY1630 derivatives containing the plc1Δ1::HIS3 allele) contain, respectively, Cadus plasmids 1443 (no PLC), 1637 (GAL1p-rat PLC-β1) or 1639 (GAL1p-PLC1)(See Strain List, Table 2). While all three strains grow at 30°C on both synthetic complete and rich media, CY1904, harboring a GAL1p-PLC1 expression cassette on a 2 micron plasmid, grows better than either CY1901 (empty vector) or CY1903 (harboring a GAL1p-rat PLC-β1 expression cassette on a 2 micron plasmid). At

non-permissive temperatures (>35°C). CY1901 and CY1903 failed to form colonies after 3 days in culture, while CY1904 forms large colonies.

CY1901, CY1903, and CY1904 (all haploid strains) were transformed by lithium acetate transformation procedures (Clontech, as per manufacturer's recommendations) with a panel of low-copy number (CEN ARS) Gα subunit expression plasmids coding for the following genes under the transcriptional control of the GPA1 upstream promoter: Cadus plasmid 1127 (empty vector), 1179 (GPA1), 1181 (GαS), 1606 (Gαi2), 1617 (Gα16), 1753 (Gα16-Q209L GTPase⁻ activated allele), 1685 (Gαq), and 1749 (Gαq-Q209L GTPase⁻ activated allele)(See Strain List, Table 1). Following transformation into the strains listed above, isolated colonies were streaked to selective plates (-Ura, -Trp + glucose) and incubated at 30°C or at 37°C for up to 96 hours. While all strains containing all combinations of PLC expression vectors (1443, 1637, and 1639) and G-protein expression vectors grew under permissive conditions (30°C), only those strains expressing the yeast PLC1 gene under the control of the GAL1 promoter grew at 37°C. Induction of the GAL1 promoter by growth on low glucose/high galactose plates did not result in rescue of the ts phenotypes of CY1901 or CY1903 derivatives, either. Even at times up to 96 hours post-plating, there was no evidence of growth under non-permissive conditions for strains harboring vectors expressing rat PLC-β1 from the GAL1 promoter. These results indicate that plasmids encoding the rat PLC-β1 driven by the GAL1 promoter are not capable of complementing the ts defect in *plc1* strains of yeast.

CUP-PLC-β expression vectors

One possible explanation for the negative results described in the previous section is a lack of expression or an inappropriate level of expression of rat PLC-β1 from the GAL1 promoter. A second potential difficulty presented with the GAL1 promoter relates to the inability of *plc*1 strains to utilitize galactose as a carbon source. If galactose utilization is compromised in these strains, then induction of galactose sensitive promoters like GAL1p and GAL10p would also be compromised. To circumvent both of these potential problems, the mammalian PLC-β isozymes were expressed from an inducible promoter whose activation is not dependent on carbon sources other than glucose. Unlike other high level constitutive promoters (i.e. PGK or ADH1), has not been associated with cellular toxicity. This sort of toxicity has, in fact been reported in the literature by Flick and Thorner (1993) with respect to overexpression of the yeast PLC1 gene from the GAL1 promoter in the presence of galactose. Flick and Thorner report that the growth of wildtype strains of yeast that contained the PLC1 gene under the control of the GAL1 promoter was compromised

when the GAL1 promoter was induced on galactose but not when it was repressed on glucose-containing media.

Four novel PLC-β constructs were made; two encoding the rat PLC-β1 gene and two encoding the human PLC-β2 gene each under the control of the CUP1 promoter. In these constructs, the nucleotide sequences of amino terminal regions of rat PLC-β1 and human PLC-β2 were altered to reflect a bias towards yeast codon usage instead of mammalian codon usage. The amino terminal seven residues of PLC-β1 and the amino terminal 21 residues of human PLC-β2 were mutated using synthetic oligonucleotides to effect these changes. The nucleotide sequence of each construct subsequently was verified by dideoxynucleotide sequencing methods on both DNA strands.

Yeast Strain CY1630 was transformed with each of these plasmids, as well as other PLC constructs in which PLC isozymes are driven by the GAL1p or ADH1 promoters, in the presence or absence of plasmids expressing various Gα subunits. Initial experiments tested for complementation of the temperature sensitive (ts) phenotype associated with the plc1 genotype by human PLC-β2 and rat PLC-β1 in the presence of a hybrid Gα subunit composed of the amino terminal 60% of the yeast GPA1 subunit and the carboxyl terminal 40% of the human Gα16 subunit (Gα16-Bam) This hybrid construct was chosen over a variety of other human Gα subunits for two reasons. First, in independent experiments it had been demonstrated that this particular hybrid subunit is functionally expressed in yeast (i.e. it couples to yeast Gβγ subunits and supresses activation of the pheromone response pathway in gpa1- strains). Second, the structural determinants involved in effector activation by Gα subunits are thought to map to residues in the carboxyl terminal portions of Gα subunits (Berlot and Bourne (1992); Rarick et al. (1992); Artemeyer et al.(1993)).

Y1630 (plc1) derivatives (See Strain List, Table 2) harboring combinations of mammalian PLC-β isozymes and Gα subunits were first colony purified and then duplicate sibs of each co-transformation assayed for growth at 30oC and 37oC in the presence or absence of 100uM CuSO4. At 48 hours post-plating, all plc1 strains, including those containing plasmids encoding rat PLC-β1, grew at 30oC, albeit less well than strains CY1633 (PLC1) or CY1904 (plc1Δ1::HIS3 [GAL1p-PLC1]). At non-permissive temperatures, several differences could be observed between strains harboring mammalian PLC-β isozymes and those harboring empty vector by 48 hours post-plating. First, strains containing high copy versions (2 micron based) of rat-PLC-β1 grew at 37oC regardless of the promoter driving expression of the PLC-β isozyme, while strains containing empty vector did not. CY1630 derivatives containing Cadus plasmid 1640 (ADH1p-rat PLC-β1) or Cadus plasmid

1922 (CUP-human PLC-β1) formed small colonies within 48 hours at 37oC, while CY1630 derivatives containing Cadus 1443 (empty vector) did not.

Second, CY1630 derivatives co-transformed with a low copy (CEN ARS) plasmid encoding CUP-human PLC-β2 and a low copy (CEN ARS) plasmid encoding the Gα16-Bam allele grew at both 30oC and at 37oC. Third, growth of CY1630 derivatives at 37oC in this assay was not due to expression of the Gα16-Bam allele alone as strains containing the Gα subunit variant alone in the absence of rat or human PLC-β isozymes did not support growth at 37oC.

Finally, growth of CY1630 derivatives expressing mammalian PLC isozymes was unaffected by the addition of 100uM CuSO4 at 30oC, while at 37oC growth in the presence of copper was less than growth in the absence of copper. This apparent inhibition of growth in the presence of copper at non-permissive temperatures may reflect toxicity due to overexpression of phospholipase C enzymatic activity by analogy with the observations made by Flick and Thorner on overexpression of PLC1. These data represent the first demonstration to our knowledge that expression of mammalian PLC-β isozymes in yeast could functionally complement defects in the PLC1 allele of S. cerevisiae. These data also demonstrate that members of the G-protein regulated PLC-β family of phospholipase C enzymes can functionally replace members of the PLC-delta family in yeast.

In an extension of these initial observations, a series of CY1630 derivatives were constructed in which different combinations of mammalian PLC-β isozymes and mammalian Gα subunits were co-expressed. The table of yeast strains (Table 2) lists the combinations tested in growth complementation assays. Strains containing any combination of Gq family members (wildtype alleles of q, 11, 16 as well as GTPase-deficient alleles of q and 16) and PLC-β2 supported growth under non-permissive conditions. Gα subunits from the GαS and Gαi families, on the hand, did not support growth of plc1Δ strains under restrictive conditions. The Figure shows representative data. plc1Δ strains expressing PLC-β1 under the control of the CUP promoter grow under restrictive conditions regardless of the presence or absence of Gα subunits.

Complementation of the temperature sensitive and NaCl-sensitive phenotype associated with the *plc1* genotype was observed in two sets of circumstances. In *PLC*Δ strains containing high copy plasmids encoding either rat PLC-β1 (Plasmid 1922) or human PLC-β2 (Plasmid 1964), growth at 37oC did not require co-expression of a mammalian Gα subunit. These strains, therefore, display G-protein independent complementation of the ts

defect. In strains containing low copy plasmids encoding the human PLC-β2 allele (Plasmid 1961), growth at 37oC required co-expression of a Gα subunit from the Gq family. *plc1* strains containing human PLC-β2 and Gq, -11, or -16, either on low or high copy number plasmids, all grew at 37oC. Furthermore, strains expressing human PLC-β2 and activated forms of Gαq (Q209L) or Gα16 (Q212L) grew robustly at both 30oC and 37oC while strains harboring human PLC-β2 and an activated form of GαS (Q227L) failed to support growth at 37oC. 3

Example 2. Complementation of the yeast plc1 mutation by human PLC-β3

Human PLC-β3 has been found to complement the temperature-sensitive and NaCl sensitive phenotypes associated with the *plc1* genotype in yeast in a G-protein independent fashion. These conclusions are based on the finding that expression of the human PLC-β3 in yeast strains with the *plc1* background under the control of the CUP promoter on either high or low copy plasmids, results in growth on 0.5M NaCl or at elevated temperatures, independent of the co-expression of Gα subunits of the Gq family (i.e. q, 11, 14, 15, or 16). In this respect the characteristics of the human PLC-β3 isoform expressed in yeast resemble those of rat PLC-β1 in exhibiting G-protein independent growth under non-permissive conditions, as distinct from the G-protein dependent growth phenotype of the human PLC-β2 isoform.

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All of the references and publications cited herein are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Cadus Pharmaceutical Corporation.
(B) STREET: Old Saw Mill River Road
(C) CITY: Tarrytown
(D) STATE: NY
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 10591
(G) TELEPHONE: (914) 345-3344
(H) TELEFAX: (914) 345-3565

(ii) TITLE OF INVENTION: Functional Vertebrate Phospholipase C in Yeast

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII (text)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/481,632
(B) FILING DATE: 07-JUN-1995

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGCGTCTCA CATGGCCCGC TCGCTGACC 29

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGCTCGAGC TGGGTCACAG CAGGTGGATC 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGCGTCTCA CATGACTCTG GAGTCCATCA TG 32

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGCGTCTCC TCGAGGCACG GTTAGACCAG 30

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGGTCTCC CATGACTCTG GAGTCCATGA TG 32

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGCTCGAGG AAGCCTGGCC CT 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CATGGCTGGT GCTCAACCAG GTG 23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGCACACCTG GTTGAGCACC AGC 23

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CATGTCTTTG TTGAACCCAG TTTTGTTGCC ACCAAAGGTT AAGGCTTACT TGTCTCAAGG 60

TGAGC 65

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCTCACCTTG AGACAAGTAA GCCTTAACCT TTGGTGGCAA CAAAACTGGG TTCAACAAAG 60

A 61

We Claim:

1. A yeast cell comprising a heterologous gene encoding a heterolgous phospholipase C, which heterologous phospholipase C is expressed by the cell and functionally integrated into a signal pathway of the cell.

2. The cell of claim 1, wherein the heterologous phospholipase C complements a loss-of-function mutation of an endogenous phospholipase gene of the cell.

3. The cell of any of claims 2 or 30, wherein the loss-of-function mutation is *plc1*Δ.

4. The cell of any of claims 1 or 29, wherein the heterologous phospholipase C is a phospholipase selected from the group consisting of PLCα's, PLCβ's, PLCδ's and PLCγ's.

5. The cell of claim 1, wherein the heterologous phospholipase C is a phospholipase whose activity can be modulated by a heterotrimeric G protein or one or more subunits thereof.

6. The cell of claim 1, wherein the heterologous phospholipase C is a phospholipase selected from the group consisting of PLCβ1, PLCβ2, PLCβ3 and PLCβ4.

7. The cell of any of claims 2 or 29, wherein the heterologous phospholipase C is a mammalian phospholipase.

8. The cell of claim 7, wherein the heterologous phospholipase C is a human phospholipase.

9. The cell of any of claims 1, 27 or 29, which cell further comprises a heterologous gene encoding a regulatory protein, which regulatory protein modulates the activity of the heterologous phospholipase C.

10. A cell of claim 9, wherein the regulatory protein is selected from the group consisting of: profilin, cofilin, gelsolin, α actinin, a G-protein and a G protein subunit or complexes thereof.

11. The cell of claim 10, wherein the regulatory protein is a Gα or Gβγ.

12. The cell of any of claims 1 or 31, which cell further comprises a heterologous gene encoding a heterologous receptor which is capable transducing signals via the signal pathway.

13. The cell of claim 12, wherein the receptor is a G-protein coupled receptor capable of transducing a signal to the heterologous phospholipase.

14. The cell of claim 13, wherein the G-protein coupled receptor is selected from the group consisting of: a chemoattractant peptide receptor, a cytokine receptor, a neuropeptide receptor, a light receptor, a neurotransmitter receptor, a cyclic AMP receptor, and a polypeptide hormone receptor.

15. The cell of claim 14, wherein the receptor is selected from the group consisting of: an α 1A-adrenergic receptor, an α1B-adrenergic receptor, an α1C-adrenergic receptor, an M_1 ACh receptor, an M_3 ACh receptor, an M_5 ACh receptor, a D_2 dopamine receptor, a D_3 dopamine receptor, an A1 adenosine receptor, a 5HT1-like receptor, a 5HT1d-like receptor, a 5HT1d beta receptor, a substance K (neurokinin A) receptor, a f-Met-Leu-Phe (FMLP) receptor, an angiotensin II type 1 receptor, a *mas* proto-oncogene receptor, an endothelin ETA receptor, an endothelin ETB receptor, a thrombin receptor, a growth hormone-releasing hormone (GHRH) receptor, a vasoactive intestinal peptide receptor, an oxytocin receptor, a SST3 receptor, an Lutinizing hormone/chorionic gonadotropin (LH/CG) receptor, a thromboxane A2 receptor, a platelet-activating factor (PAF) receptor, a C5a anaphylatoxin receptor, an Interleukin 8 (IL-8), IL-8A receptor, an IL-8B receptor, a mip-1/RANTES receptor, a metabotropic glutamate mGlu1-5 receptor, an ATP receptor, an amyloid protein precursor receptor, a bradykinin receptor, a gonadotropin-releasing hormone receptor, a cholecystokinin receptor, an antidiuretic hormone receptor, an adrenocorticotropic hormone II receptor, LTB4 receptor, LTD4 receptor, tachykinin receptor, thyrotropin releasing hormone receptor, vasopressin receptor and oxytocin receptor.

16. The cell of claim 12, wherein the receptor protein is a receptor tyrosine kinase.

17. The cell of any of claims 1 or 31, wherein the signal pathway modulates gene expression.

18. The cell of any of claims 1 or 31, which cell further comprises a reporter gene construct containing a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the signal pathway, expression of the reporter gene providing the detectable signal.

19. The cell of claim 18, wherein the reporter gene encodes a gene product that gives rise to a detectable signal selected from the group consisting of: color, fluorescence, luminescence, cell viability relief of a cell nutritional requirement, cell growth, and drug resistance.

20. The cell of claim 19, wherein the reporter gene encodes a gene product selected from the group consisting of chloramphenicol acetyl transferase, β-galactosidase, alkaline phosphatase, β-lactamase, luciferase and green fluorescent protein.

21. The cellof claim 19, wherein the reporter gene encodes a gene product which confers a growth advantage.

22. The cell of claim 19, wherein the reporter gene encodes a gene product which confers on the cell the ability to grow in media containing canavanine or a similar selective agent.

23. The cell of any of claims 1 or 31, wherein the signal pathway modulates calcium mobilization.

24. The cell of any of claims 1 or 31, wherein the signal pathway modulates PKC activity.

25. The cell of any of claims 1 or 31, wherein the signal pathway modulates temperature sensitivity of growth of the cell.

26. The cell of any of claims 1 or 29, wherein the heterologous phospholipase C is constutitively activated.

27. A yeast cell comprising a first heterologous gene encoding a mammalian β-type phospholipase C, which phospholipase C is expressed by the cell and is capable of hydrolyzing phosphatidylinositol 4,5-bisphosphate.

28. The cell of claim 27, wherein the phospholipase C is selected from the group consisting of PLCβ1, PLCβ2, PLCβ3 and PLCβ4.

29. A yeast cell comprising:

i) a phospholipase gene having a mutation thereto which confers a first detectable phenotype on the cell; and

ii) a first heterologous gene encoding a polypeptide having a phospholipase C activity, which heterologous phospholipase C activity modulates the mutation and confers a second detectable phenotype on the cell.

30. The cell of claim 29, wherein the mutation is a loss-of-function mutation, and the heterologous phospholipase C complements the loss-of-function.

31. The cell of claim 29, wherein the heterologous phospholipase C activity is functionally integrated in a signal pathway of the cell.

32. The cell of claim 29, wherein the first detectable phenotype is selected from the group consisting of temperature sensitive growth and NaCl-sensitivity.

33. The cell of any of claims 1, 27 or 29, which cell is a *Saccharomyces* cell.

34. A variegated culture comprising the cells of any of claims 1, 27 or 29, wherein the cells further comprise an expressible recombinant gene encoding a heterologous test polypeptide, and a mixture of the cells in the culture collectively express a variegated population of test polypeptides.

35. An assay for identifying a modulator of a phospholipase C activity, comprising the steps of:

i) contacting the cell of claim 1 with a test compound under conditions appropriate for detecting an intracellular signal transduced via the signal pathway; and

ii) measuring a level of signal transduced by the signal pathway in the presence of the test compound,

wherein a statistically significant difference in the level of signal in the presence of the test compound, relative to the absence of test compound, indicates that the test compound is a modulator of the heterologous phospholipase C activity.

36. The assay of claim 35, wherein the intracellular signal is detected by expression of a reporter gene operably linked to transcriptional regulatory elements sensitive to the signal pathway.

37. The assay of claim 35, wherein the intracellular signal is detected by measuring products of the hydrolysis of phosphatidylinositol 4,5-bisphosphate.

38. The assay of claim 35, wherein the intracellular signal is detected by measuring one or more of the degree of Ca^{2+} mobilization and the activation of a protein kinase.

39. The assay of claim 35, wherein the intracellular signal is detected by measuring a change in growth.

40. An assay for identifying a modulator of a phospholipase C activity, comprising the steps of:

i) contacting the cell of claim 29 with a test compound under conditions appropriate for detecting one or both of the first and second phenotypes;

ii) measuring the level or degree of the first and/or second phenotype in the presence of the test compound; and

iii) comparing the measurement of step ii) with a similar measurement in the absence of the test compound or the absence of the heterologous phospholipase C,

wherein a statistically significant change in the measured phenotype in the presence of the test compound, relative to the absence of test compound or phospholipase C, indicates that the test compound is a modulator of the heterologous phospholipase C activity.

41. An assay for identifying a modulator of a phospholipase C activity, comprising the steps of:

i) contacting the cell of claim 18 with a test compound under conditions appropriate for expresion of the reporter gene;

ii) detecting expression of the reporter gene in the presence of the test compound; and

iii) comparing the level of reporter gene expression in the presence of the test compound to the level of reporter gene expression in the absence of the test compound or the absence of the heterologous phospholipase C,

wherein a statistically significant difference in the level of reporter gene expression in the presence of the test compound, relative to the absence of test compound or phospholipase C, indicates that the test compound is a modulator of the heterologous phospholipase C activity.

42. A differential screening assay for identifying an agent which selectively inhibits a non-mammalian phospholipase C, comprising the steps of:

i) providing a first yeast cell containing a non-mammalian phospholipase C gene;

ii) providing a second yeast cell expressing a mammalian phospholipase C gene;

iii) contacting each of the first and second yeast cells with a test compound;

iv) detecting or quantitating the activity of the phospholipases of the first and second cell in the presence of the test compound,

wherein, a statistically greater decrease in the phospholipase activity of the first cell, relative to the second cell, indicates that the test compound selectively inhibits the non-mammalian phospholipase C.

43. The assay of claim 42, wherein the phospholipase C of the first yeast cell is a vertebrate phospholipase C, and the phospholipase C of the second yeast cell is a phospholipase C from a pathogen of the vertebrate.

44. The assay of claim 43, wherein the vertebrate is a human.

45. The assay of claim 43, wherein the human pathogen is selected from the group consisting of a fungus, virus, bacteria and protozoan.

46. The assay of claim 43, wherein the human pathogen is human fungal pathogen.

47. The assay of any of claims 35, 40, 41 or 42, wherein the assay is repeated for a library of at least 100 different test compounds.

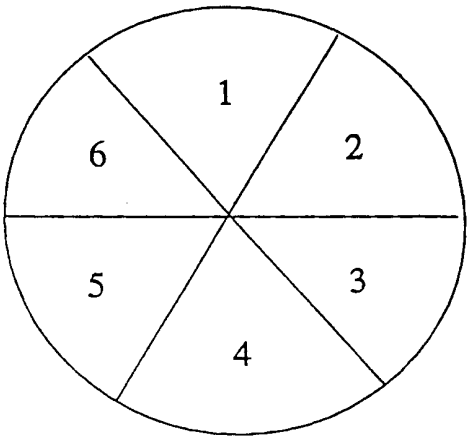
48. The assay of any of claims 35, 40, 41 or 42, wherein the test compound is selected from the group consisting of small organic molecules, and natural product extracts.

49. A collection of at least two different yeast cells, each cell comprising a gene encoding a different phospholipase

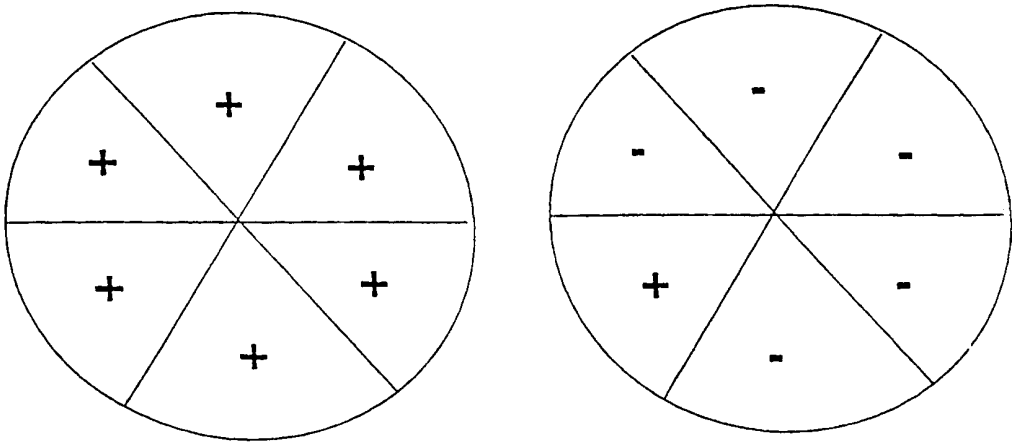
50. The collection of claim 49, wherein each different yeast cell encodes a different isotypes of phospholipase obtained from the same organism or the same or different isotype obtained from different organisms.

5

Figure Human PLC-β2 and Gα16 Complement *plc1*- Growth Defects on High Salt



SC medium (1.7 mM NaCl) **SC + 0.5M NaCl**



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(57) Abstract

Yeast cells comprising a heterologous phospholipase C protein and methods for their use in drug screening assays to identify a modulator (i.e. agonist or antagonist) of a phospholipase C are described.

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